The Role of Notch Signalling in the Induction of Immune Responses

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Meinen Eltern

(To my parents)

DECLARATION

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree. All the work presented in this thesis, was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

Karen Wahl

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ABSTRACT

Notch signalling is implicated in a wide range of developmental processes such as neurogenesis, haematopoiesis and T cell development. Peripheral T cells continue to express Notch components suggesting a role for them also after their development. Furthermore, activation of the Notch pathway can be modulated by the presence of cytokines, induce proliferation and influence the decision between cell survival and apoptosis. These events are crucial for the induction and modulation of an immune response.

Therefore the expression and the role of Notch receptors and ligands during the induction of an immune response were investigated. I have shown that components of the Notch pathway were present in peripheral CD4 and CD8 T cells. Upon culturing and activation *in vitro*, many Notch components were differentially expressed, in particular targets of Notch activation. In contrast, bone marrow-derived dendritic cells (DCs) expressed only low levels of Notch targets. However, the Notch ligand Jagged1 was strongly upregulated upon maturation of DCs with TNFα or LPS.

By regulating the expression of the ligands, a role for antigen presenting cells (APCs) as the "signalling cells" is likely, whereas T cells expressing Notch receptors and Notch target genes may be the "receiving cells". To investigate this hypothesis *in vivo*, transgenic mice with inducible overexpression of Jagged1 or Delta1 in DCs were generated.

An *in vitro* approach to study the role of Notch ligands on APCs was carried out using an I-A^b transfected murine fibroblast cell line (I-A^{b+} L cells) with endogenous B7.1 expression. I-A^{b+} L cells co-transfected with Jagged1 or Delta1 (Jagged1$^+$ or Delta1$^+$ L cells, respectively) were used as APCs in a mixed lymphocyte reaction (MLR) *in vitro*. Jagged1$^+$ and I-A^{b+} L cells induced similar levels of allogeneic T cell proliferation, whereas Delta1$^+$ L cells had a slightly increased capacity to activate T cell proliferation. There were no phenotypical differences or changes in the level of apoptosis observed between T cells activated by Jagged1$^+$, Delta1$^+$ or I-A^{b+} L cells. However, IFNγ secretion by T cells in response to Jagged1$^+$ L cells was strongly reduced, whereas Delta1$^+$ and I-A^{b+} L cells induced normal levels of IFNγ secretion. There were no significant differences neither in the level of intracellular IFNγ nor of IFNγ transcripts between T cells in response to Jagged1$^+$, Delta1$^+$ or

I-A^{b+} L cells. Therefore I hypothesise that Jagged1-induced Notch signalling may be involved at the level of IFNγ secretion.

In summary I have shown that Notch components are expressed and differentially regulated in DCs and T lymphocytes and that APCs can act as the "signalling" cell by activating the Notch pathway in the "receiving" T cells. Jagged1 expressed on APCs led to decreased secretion of IFNγ by T lymphocytes during a MLR. These findings may have important implications in the treatment of autoimmune diseases or in transplantation.

ABBREVIATIONS

[α^{33}P]-ATP	^{33}phosphorous-deoxy-A-triphosphate
[γ-^{35}S]-ATP	^{35}sulphur-deoxy-A-triphosphate
γc	common gamma chain
7-AAD	7-aminoactinomycin D
A	adenosine
Ac-Sc	Achaete-Scute complex
AMV	*avian myeloblastosis virus*
APC	antigen presenting cell
bHLH	basic helix-loop-helix
bp	base pairs
BSA	bovine serum albumin
BM	bone marrow
C	cytidine
C. elegans	*Caenorhabditis elegans*
CBF1	C promoter binding factor 1
CD	cluster of differentiation
CD40L	CD40 ligand
cDNA	complementary DNA
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CR	complement receptor
CSL	CBF1, Su(H), LAG-1
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
Cy	Cy-Chrome
DAG	diacylglycerol
DC	dendritic cell
DCIR	DC immunoreceptor
DC-SIGN	DC-specific ICAM-grabbing non-integrin
dCTP	deoxy-C-triphosphate
ddNTP	dideoxynucleoside triphosphate
DEPC-treated water	diethyl pyrocarbonate-treated water
Der p	*Dermatophagoides pteronyssinus*
Der p 1	Der p type 1 allergen
dGTP	deoxy-G-triphosphate
DN	$CD4^-$ $CD8^-$ double negative
DNA	deoxy ribonucleic acid
DNAse	deoxyribonuclease
dNTP	dioxynucleoside triphosphate
dox	doxycyclin
DSL	Delta/Serrate/Lag-2
DTT	dithiothreitol
dTTP	deoxy-T-triphosphate
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EBNA2	EBV nuclear antigen 2

E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid-disodium salt
EGFP	enhanced green fluorescent protein
ELC	Epstein-Barr virus-induced molecule 1 ligand chemokine
ERK	extracellular signal receptor regulated kinase
E(spl)	Enhancer of split
FACS	fluorescence-activated cell sorting
FAM	6-carboxyfluorescein
Fig.	Figure
FITC	fluorescein isothiocyanate
G	guanosine
G-CSF	granulocyte colony-stimulating factor
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
hrs	hours
HDAC	histone deacetylase complex
HDM	house dust mite
Hes	hairy/enhancer of split
HPRT	hypoxanthine phosphoribosyltransferase
HRP	horseradish peroxidase
ICAM	intercellular adhesion molecule
ICN	intracellular, activated form of Notch
IRF	interferon regulatory factor
Ig	immunoglobulin
IL-	interleukin
IL-2R	IL-2 receptor
IP_3	inositol trisphosphate
IRAK	IL-1R associated kinase
ITAM	immuno-receptor tyrosine-based activation motif
JAK	Janus kinase
JNK	Jun N terminal kinase
LC	Langerhans cell
LFA-1	leukocyte function antigen-1
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
MACS	magnetic cell sorting
MAML1	Mastermind-like-1
MAPK	mitogen-activated protein kinase
MASH1	mammalian achaete-scute homologue-1
MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
mH_2O	millipore water
MHC	major histocompatibility complex
min	minutes
MIP	macrophage inflammatory protein
mRNA	messenger RNA
NCR	Notch cytokine response
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor κB
NK	natural killer cell

nm	nanometers
PAMPs	pathogen-associated molecular patterns
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PE	phycoerythrin
PG	prostaglandin
PHA	phytohaemagglutinin
PI	phosphoinositide
PKC	protein kinase C
PLCγ	phospholipase Cγ
PMA	phorbol 12-myristate 13-acetate
poly(A)	polyadenylic
PRR	pattern-recognition receptor
PS	phosphatidylserine
PTK	protein tyrosine kinase
RAM	RBP-J association molecule
RANK	receptor activator of NF-κB
RANTES	regulated upon activation, normal T cell expressed and secreted
RBC	red blood cells
RBP-Jκ	recombination signal binding protein Jκ
RNA	ribonucleic acid
RNAse	ribonuclease
rmGM-CSF	recombinant murine GM-CSF
RT	reverse transcriptase
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
rtTA	reverse tetracycline-controlled transactivator
SLC	secondary lymphoid tissue chemokine
SLP-76	src homology 2 domain-containing leukocyte phosphoprotein of 76kD
SOCS	suppressor of cytokine signalling
SP	single-positive
STAT	signal transducer and activator of transcription
T	thymidine
TAMRA	6-carboxytetramethylrhodamine
Tc	tetracycline
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethylethylenediamine
Tfl	*Thermus flavus*
TGF	transforming growth factor
TLE	transducin-like E(spl)
TLR	Toll-like receptor
TRAF	TNF receptor associated factor
TRANCE	TNF-related activation induced cytokine
TRE	Tc-responsive element
UV	ultraviolet
wt	wild-type
ZAP-70	zeta/ζ associated protein of 70kD

I Introduction

The immune system has developed to protect organisms against invading pathogens. It can be divided into the non-specific innate immune system and the specific adaptive immune response. Invertebrates are protected only by innate immunity. This includes anatomical (e.g. the skin) and physiological barriers (e.g. extreme pH values), soluble factors (e.g. antibacterial enzymes, interferons and the complement system), as well as phagocytes and natural killer cells (NKs). In addition to the innate immune system, vertebrates have an adaptive immune system. The major differences to the innate immune system are antigen specificity, immunological memory and the ability to discriminate between self and non-self.

Two main groups of cells are required for an effective adaptive immune response: Lymphocytes and antigen presenting cells (APCs). Lymphocytes are leukocytes that develop during haematopoiesis in the bone marrow and afterwards circulate through the lymph and blood system. There are two groups of antigen-specific lymphocytes: B cells and T cells (Raff, 1971). A variety of cells can function as APCs. The distinguishing feature of APCs is their ability to express gene products encoded by the class II major histocompatibility complex (MHC) and to deliver costimulatory signals. APCs present antigenic peptides bound to the MHC molecules. Antigens coming from intracellular replicating agents (e.g. viruses) are presented to $CD8^+$ T cells by MHC class I molecules, whereas antigens from extracellular agents (e.g. bacteria) are presented to $CD4^+$ T cells in the context of MHC class II molecules (reviewed in Androlewicz, 2001; Pieters, 2000; Thery and Amigorena, 2001). Three cell types are classified as professional APCs: dendritic cells (DCs), macrophages and B lymphocytes. These cells differ in their mechanisms of antigen uptake, in whether they constitutively express class II molecules and in their costimulatory activity.

I will discuss the development and function of DCs in detail as the work on APCs presented in my thesis has focused on this cell type; this will be followed by a brief overview of T cell activation and a more detailed review of the Notch signalling pathway.

I.1 Dendritic cells

I.1.1 Development of DCs from haematopoietic precursors

DCs arise from haematopoietic progenitor cells with myeloid differentiation potential. They can be differentiated *in vitro* from human monocytes by incubation with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 (Romani *et al.*, 1994; Sallusto and Lanzavecchia, 1994; Zhou and Tedder, 1996). Myeloid-committed precursors from murine bone marrow (BM) give rise to granulocytes, monocytes and myeloid DCs under the influence of GM-CSF (Inaba *et al.*, 1992a; Scheicher *et al.*, 1992). This suggests that DCs belong to the myeloid lineage. However, common lymphoid and common myeloid progenitors (CLP and CMP, respectively) have been identified in murine BM (Akashi *et al.*, 2000; Kondo *et al.*, 1997). IL-7 receptor α chain (IL-7Rα) expression is a main marker to distinguish these two progenitors. CLPs are IL-7Rα$^+$ and generate all lymphoid cells and certain DCs, but not myeloid or erythroid cells (Kondo *et al.*, 1997). CMPs are IL-7Rα$^-$ and give rise to precursors for megakaryocytes/erythrocytes, granulocytes, macrophages and DCs (Akashi *et al.*, 2000; Traver *et al.*, 2000). In conclusion, DCs can arise from both myeloid and lymphoid precursors, but the picture remains confused.

After production in the bone marrow, DC precursors of the myeloid line enter non-lymphoid tissues where they reside transiently before migrating to secondary lymphoid tissues. In contrast, lymphoid DC progenitors probably enter the thymus and develop into DCs that play a crucial role in the induction of thymic (central) tolerance. Mouse thymic DCs and a subpopulation of DCs in spleen and lymph nodes express several markers of lymphoid cells, such as CD8α, CD2 and CD25 (Vremec *et al.*, 1992; Wu *et al.*, 1995). Unlike the bone marrow precursors, which can produce both CD8α$^+$ and CD8α$^-$ DC populations in mouse spleen, the intrathymic precursor population only generates CD8α$^+$ DCs (Wu *et al.*, 1996) suggesting that they represent a lymphoid-related DC lineage (Fairchild and Austyn, 1990; Wu *et al.*, 1996). The similarity in phenotype between thymic DCs and CD8α$^+$ DCs from spleen and lymph nodes (LNs) suggests a common origin (Vremec and Shortman, 1997). However, recent publications have discussed the controversial origin of lymphoid DCs, with some reports suggesting that myeloid-derived DCs and lymphoid DCs originate from the same

precursor (Merad *et al.*, 2000; Traver *et al.*, 2000). Table I.1 summarises features of lymphoid and myeloid-derived DCs.

In humans, at least two subsets of DC precursors circulate in the blood: $CD14^+$ $CD11c^+$ monocytes and $CD4^+$ IL-$3R\alpha^+$ $CD11c^-$ precursor DCs (Grouard *et al.*, 1997; Olweus *et al.*, 1997; Romani *et al.*, 1994; Zhou and Tedder, 1996). Although there is controversy as to whether the latter DC precursors are myeloid or lymphoid in origin, this human DC subset is often referred to as 'lymphoid' or 'plasmacytoid' DCs. A third precursor cell type, $CD1^+$ $CD11c^+$ cells, has also been described in blood, which quickly acquires Langerhans cell characteristics if cultured in the presence of GM-CSF, IL-4 and transforming growth factor (TGF)-β (Ito *et al.*, 1999). Lymphoid precursors in the thymus giving rise to DCs, T lymphocytes and NKs were also identified in the human system (Res *et al.*, 1996). A large body of literature has recently accumulated concerning the origin and differentiation pathways of human DCs (reviewed in Banchereau *et al.*, 2000; Bell *et al.*, 1999; Liu, 2001). However, my thesis will concentrate mainly on murine DC development and function.

Table I.1. Murine DC subsets in secondary lymphoid organs.*

Postulated lineage	**Lymphoid**	**Myeloid**	
	$CD8\alpha^+$ DCs	$CD8\alpha^-$ DCs	LC-derived DCs
Phenotype			
Precursor	IL-$7R\alpha^+$	IL-$7R\alpha^-$	?
Mature DCs	$CD11c^+$	$CD11c^+$	$CD11c^+$
	MHC class II^+	MHC class II^+	MHC class II^+
	$CD8\alpha^+$	$CD8\alpha^-$	$CD8\alpha^{+/-}$ (?)
	$CD4^-$	$CD4^+$ (subsets)	$CD4^-$
	DEC-205^+	DEC-$205^{dull/-}$	DEC-205^+
	$CD11b^{dull/-}$	$CD11b^+$	$CD11b^+$
	$CD40^+$	$CD40^+$	$CD40^+$
	$B7.2^+$	$B7.2^+$	$B7.2^+$
	Birbeck $granule^-$	Birbeck $granule^-$	Birbeck $granule^+$
Localisation	T cell zones of lymphoid organs, thymic cortex	Marginal zones of spleen (move to T cell zones when activated); sub-capsular sinus of LNs?	Immature LCs in epithelia; LC-derived DCs in T cell zones of LNs
Function			
Antigen capture	+	++	++
Antigen processing	++	++	++
IL-12 secretion	++++	+/–	++++
IFNγ secretion	++++	–	?
$CD4^+$ T cell polarisation	T_H1	T_H0/T_H2	?

* This is a simplified summary and cannot include all DC subsets and functions. Based on Pulendran *et al.*, 2001)

I.1.2 DC populations

The epidermis contains a special subset of DCs called **Langerhans cells (LCs)** (Table I.1; Davis *et al.*, 1988). They contain Birbeck granules and are characterised by the expression of CD1a and the cutaneous lymphocyte-associated antigen (CLA) (Davis *et al.*, 1988; Koszik *et al.*, 1994). $CD1a^+$ DCs have also been identified in other epithelial surfaces such as the urothelium and gut epithelium (Hussain and Lehner, 1995; Troy *et al.*, 1998). The relationship between murine LCs and lymphoid and myeloid DCs is not completely understood.

Mouse peripheral blood mononuclear cells generate DC-like cells when cultured in GM-CSF (Inaba *et al.*, 1992b), suggesting circulating DC precursors. These are destined to provide **interstitial DCs** in non-lymphoid tissues (Matsuno *et al.*, 1996). In humans, several subsets of blood DCs have been identified (Egner *et al.*, 1993; Olweus *et al.*, 1997). Interstitial DCs are found in almost all organs including liver (Prickett *et al.*, 1988), kidney (Hart *et al.*, 1981), heart (Spencer and Fabre, 1990) and other connective tissue (Hart and Fabre, 1981). Like the epithelial associated LCs, interstitial DCs are believed to form a sentinel network of antigen-receptive cells, which subsequently move antigen centrally to provide activated APCs and generate T lymphocyte responses.

Tissue damage/inflammation, so-called "danger" signals (Matzinger, 1994), mobilise tissue DCs to migrate from skin, other non-lymphoid interstitial sites and the mucosal surfaces into the afferent lymph, where they are recognised as **veiled cells** (Bujdoso *et al.*, 1989; Roake *et al.*, 1995a). Peripheral DCs migrate as veiled cells towards afferent lymphatics and traffic in the draining lymph to the lymph nodes (LNs) where they enter the T lymphocyte-rich paracortical areas (Fig. I.1; Fossum, 1988).

It has been assumed (there have been few direct comparisons) that DCs isolated from intact lymphoid tissues (Steinman *et al.*, 1974) represent the **interdigitating DCs** observed by immunohistochemistry, light and electron microscopy (Veerman and van Ewijk, 1975). Phenotypically distinct populations of interdigitating DCs are found in the mouse spleen. Double staining for CD8α and CD11c identified two DC subsets (Suss and Shortman, 1996), whereas staining of splenocytes of Flt-3L treated mice for CD8α and CD11b divided DCs into five distinct subsets (Maraskovsky *et al.*, 1996). This complexity suggests that stages of activation contribute to the phenotypic diversity which will be discussed later.

I.1.3 Recruitment

Lipopolysaccharide (LPS), GM-CSF, IL-6 and probably other stimuli recruit DC precursors from the blood to the tissues (Fig. I.1; Kaplan *et al.*, 1992; Roake *et al.*, 1995b). The basal rate of tissue entry is boosted 5- to 10-fold by "danger" signals such as pathogens, inflammation, tissue damage and necrosis (Matzinger, 1994). DCs accumulate rapidly (within an hour) at the sites of antigen (McWilliam *et al.*, 1994). This accumulation likely represents recruitment of circulating DC precursors, in response to the production of classical chemoattractants (formyl peptides and complement derivative C5a) and of chemokines upon local inflammation (Adams and Lloyd, 1997; Sallusto and Lanzavecchia, 2000; Sozzani *et al.*, 1995). Chemokines play a role not only in extravasation, but also in the subsequent process of migration within the tissues to the final target. Different chemokines provide codes for areas that are undergoing different types of inflammatory reactions or are sites of constitutive traffic. A current view is that the whole migration process occurs in distinct steps, each driven by a particular chemokine-chemokine receptor pair (Table I.2; Foxman *et al.*, 1997).

DCs provide a striking example of this so-called multistep navigation, since they use different sets of chemokine receptors to migrate from blood to inflamed tissues and from there to the lymphatics by which they reach their final destination in the T cell areas of lymph nodes. However, the expression of a particular receptor does not always indicate that DCs demonstrate chemotaxis in response to all chemokines that bind that receptor (Sozzani *et al.*, 1995). Besides being attracted by inflammatory chemokines, DCs also produce these chemokines in large amounts. The production of chemokines by DCs is rapidly triggered after exposure to various maturation stimuli and/or upon encounters with T cells, which can take place not only in the secondary lymphoid organ but also at the site of tissue injury. This enhances recruitment of lymphocytes and immature DCs, which is important to sustain antigen sampling at later time points and downregulates the expression of the cognate receptors on maturing DCs, thus allowing them to leave the inflamed tissues (Dieu *et al.*, 1998; Sallusto *et al.*, 1998; Sozzani *et al.*, 1999; Tang and Cyster, 1999).

Table I.2. Expression of chemokine receptors by dendritic cells.

Receptor	Ligands
Immature DCs*	
CCR1	MIP-1α, RANTES, MCP-3, MIP-5
CCR2	MCPs
CCR4	TARC, MDC
CCR5	MIP-1α, MIP-1β, RANTES, MCP-2
CCR6	MIP-3α/LARC
CXCR1	IL-8
CXCR4	SDF-1
Mature DCs	
CCR7	MIP-3β/ELC, SLC

* Note that expression of chemokine receptors on immature DCs also depends on the DC subset.
ELC: Epstein-Barr virus-induced molecule 1 ligand chemokine; LARC: liver and activation regulated chemokine; MIP: macrophage inflammatory protein; RANTES: regulated on activation, normal T expressed and secreted; MCP: monocyte chemoattractant protein; TARC: thymus and activation-regulated chemokine; MDC: macrophage-derived chemokine; SDF: stromal-derived factor; SLC: secondary lymphoid-tissue chemokine. Based on Cascieri & Springer (2000).

During their migration, DCs are involved in several adhesion events. For instance, E-cadherin is present on both mouse and human LCs and is downregulated upon antigen encounter allowing LC migration out of the skin (Blauvelt *et al.*, 1995; Tang *et al.*, 1993). Although selectins have not yet been described on DCs, blood DCs do express a glycosylated form of P-selectin glycoprotein ligand-1 (PSGL-1) that binds to P- and E-selectins, which are expressed at low levels on endothelial cells and are upregulated by inflammatory stimuli (Adams *et al.*, 1996; Robert *et al.*, 1999). LCs express the E-selectin ligand CLA (Koszik *et al.*, 1994), which may be involved in cell interaction during trafficking. Interaction between DCs and the connective tissue is stabilised by isoforms of the CD44 molecule, which is expressed in high density by DCs (Sallusto and Lanzavecchia, 1994).

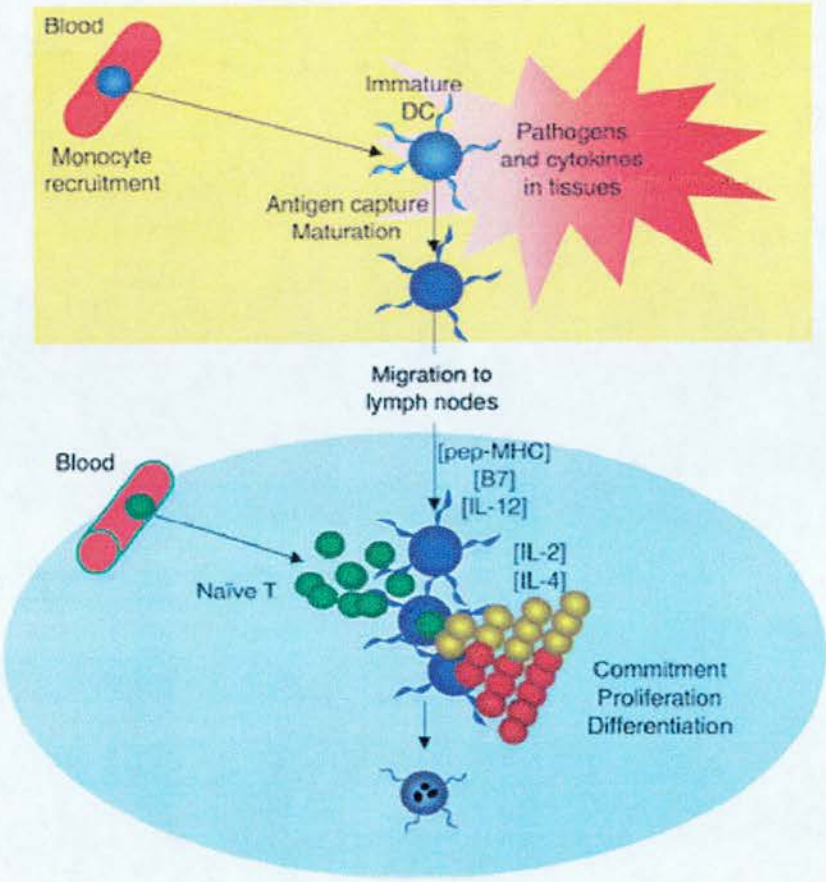


Figure I.1. T cell priming by DCs.
Recruitment of DC precursors (monocytes) into peripheral tissues and maturation of DCs in response to pathogens or cytokines result in migration to the draining lymph node of large numbers of DCs carrying high levels of peptide-MHC complexes and B7. By physical interaction through an immunological synapse, naïve T cells (green) achieve stimulation and become committed to proliferate in response to IL-2. Sustained TCR stimulation by continuous contact with DCs and polarising cytokines (IL-12 and IL-4) promote T cell differentiation to non-lymphoid tissue-homing effector cells (red). T cells receiving a shorter stimulation do not acquire effector function and retain lymph node-homing capacity (yellow). Mature DCs have a short life-span and die within 2-3 days. As a consequence, the composition of DCs in the lymph nodes dynamically reflects what is occurring in the peripheral tissues. Reprinted from Lanzavecchia & Sallusto (2001a) with permission from Elsevier Science.

I.1.4 Antigen capture and presentation

Immature DCs are very efficient in antigen uptake and can use several pathways, such as macropinocytosis, phagocytosis and receptor-mediated endocytosis.

Macropinocytosis is an actin-driven endocytic process in which plasma membrane fuses to form vesicles that non-selectively include solutes and antigens present in the fluid phase (Sallusto *et al.*, 1995). This process allows cells to continually take up large amounts of extracellular fluid containing soluble antigens. Immature DCs are particularly active at macropinocytosis, accumulating nearly an entire cell volume over one-hour period (Sallusto *et al.*, 1995).

Phagocytosis is a process by which particles, microbes or fragments of dead cells are engulfed and internalised, usually by specific membrane receptors. The process is actin-mediated and requires the action of a number of small GTP-binding proteins (Franc *et al.*, 1999). Fc receptors are involved in the uptake of antibody-coated bacteria by macrophages. Antibody-mediated phagocytosis of bacteria has not been directly shown for DCs, even though two Fcγ receptors, FcγRI (CD64) and FcγRII (CD32), are found on fresh blood DCs (Fanger *et al.*, 1996). Complement receptors can stimulate phagocytosis of C3bi-opsonised bacteria, either alone or together with Fc receptors (Carroll, 1998). Some complement receptors (CRs) have been described on DCs: CD11b and CD11c, which are subunits of CR3 and CR4, respectively, are found on several DC subsets (Egner and Hart, 1995; Metlay *et al.*, 1990; Shibaki *et al.*, 1995). Receptors suggested to mediate uptake of apoptotic bodies by DCs are certain integrin receptors and the scavenger receptor CD36 (Bellone, 2000; Larsson *et al.*, 2001).

During **endocytosis**, soluble antigens can be captured by a wide variety of specific receptors, which are then internalised in clathrin-coated vesicles. The mechanism of uptake is distinct from phagocytosis or macropinocytosis, in that it does not require actin filaments, but does depend on certain Ras proteins (Cox *et al.*, 2000). In addition to Fcγ receptors, immature DCs also express the high-affinity receptor for IgE (FcεRI) as well as the low-affinity receptor FcεRII (CD23), which can induce immune responses to allergens (Bieber *et al.*, 1989; Kahlert *et al.*, 2000; Maurer *et al.*, 1996). The pattern-recognition receptors (PRRs) bind a range of molecules with structural patterns common to the surface of many microorganisms (so called pathogen-associated molecular patterns, PAMPs) but which are absent from the surface of

mammalian cells (reviewed in Akira *et al.*, 2001). PAMPs include LPS, lipoproteins, peptidoglycan and lipoteichoic acids, which are all made by bacteria, but not by eukaryotic cells. Several members of the Toll-like receptor (TLR) family are expressed on DCs (Muzio *et al.*, 2000). TLR4 plays a crucial role in LPS signalling (Poltorak *et al.*, 1998), which leads to DC activation (see below). Another family of receptors important for the recognition and uptake of complex carbohydrates are lectin-like molecules. Human monocyte-derived DCs express the macrophage mannose receptor (Sallusto *et al.*, 1995), the DC-specific ICAM-grabbing non-integrin (DC-SIGN) (Geijtenbeek *et al.*, 2000) and the DC immunoreceptor (DCIR) (Bates *et al.*, 1999). Expression of these molecules on other DC populations remains to be established. The DEC-205 molecule is related to the macrophage mannose receptor and probably acts as an antigen receptor (Jiang *et al.*, 1995). It is present on both mouse and human DCs (Guo *et al.*, 2000; Jiang *et al.*, 1995).

Soluble and particulate antigen are efficiently captured by immature DCs and targeted to MHC class II compartments (reviewed in Pieters, 2000). DCs constantly accumulate MHC class II molecules in lysosome-related intracellular compartments identified as MHC class II-rich compartments (MIICs). Whereas, in immature DCs, class II molecules are rapidly internalised and have a short half-life, maturation/inflammatory stimuli lead to a burst of class II synthesis and translocation of MHC II-peptide complexes to the cell surface where they remain stable for days and are available for recognition by $CD4^+$ T cells. To generate $CD8^+$ cytotoxic T cells (CTLs), DCs present antigenic peptides on MHC class I molecules, which can be loaded through both an endogenous and an exogenous pathway (reviewed in Androlewicz, 2001; Thery and Amigorena, 2001). The endogenous MHC class I pathway operates through the degradation of cytosolic proteins and the loading of peptides onto newly synthesised MHC class I molecules within the endoplasmic reticulum. DCs are also capable of transporting exogenous antigens from the endocytic compartment to the cytosol, leading to "cross-presentation" on MHC class I molecules to $CD8^+$ T cells (Albert *et al.*, 1998a; Androlewicz, 2001).

I.1.5 Migration to draining LN and maturation

Activation of DCs by a "danger" signal triggers immature DC to undergo phenotypic and functional changes that culminate in the complete transition from antigen-capturing cell to APC (Matzinger, 1994). However, a small fraction of tissue DCs "spontaneously" mature and migrate to the draining lymph nodes, carrying apoptotic antigens taken up in peripheral tissues (Huang *et al.*, 2000). DC maturation is a continuous process initiated in the periphery upon antigen encounter and/or inflammatory cytokines and completed during the DC-T cell interaction. For simplicity, molecules involved in subsequent activation of T cells are discussed later.

Maturation of DCs turns off their response to chemokines specific for immature DCs through either receptor downregulation or receptor desensitisation dependent on autocrine chemokine production. Maturing DCs upregulate CCR7, which recognises secondary lymphoid tissue chemokine (SLC) and Epstein-Barr virus-induced molecule 1 ligand chemokine (ELC/MIP-3β), both present in LNs (Gunn *et al.*, 1998; Sallusto *et al.*, 1998). Therefore, CCR7 is thought to encourage migration of DCs to secondary lymphoid organs. Morphological changes accompanying DC maturation include a loss of adhesive structures, cytoskeleton reorganisation, and acquisition of high cellular motility (Winzler *et al.*, 1997). Maturing DCs rapidly lose endocytic activity, increase surface expression and stability of MHC class I- and class II-peptide complexes, upregulate surface expression of adhesion and costimulatory molecules such as CD40, ICAM-1, B7.1 (CD80) and B7.2 (CD86) and secrete pro-inflammatory cytokines such as IL-1, IL-6, IL-12, IL-18 and IL-23. The involvement of these molecules in T cell activation and/or polarisation is discussed in the following sections.

I.2 DC-T cell interaction

I.2.1 T cell priming

The ability to prime naïve $CD4^+$ T cells constitutes a unique and critical function of DCs both *in vitro* and *in vivo*. DCs pulsed with antigen elicit potent $CD4^+$ T helper (T_H) responses when injected into mice suggesting direct antigen presentation (Inaba *et al.*, 1990). In the presence of soluble antigen, T_H cells primed by DCs can interact with B cells and stimulate antigen-specific antibody production (Sornasse *et al.*, 1992). DCs are equally important in priming naïve $CD8^+$ T cells. *In vitro*, DCs can stimulate the proliferation of allogeneic $CD8^+$ T cells in the absence of T cell help (Young and Steinman, 1990). However, for the *in vivo* generation of CTL responses to exogenous antigen, which must be taken up, processed and presented on class I MHC (a process termed cross-presentation), the DCs require to be "licensed" to activate $CD8^+$ T cells by T helpers via upregulation of CD40 on the DCs (Bennett *et al.*, 1998; Schoenberger *et al.*, 1998). Thus, a "licensed" or "conditioned" DC becomes a transient bridge between a T_H cell and a CTL (Ridge *et al.*, 1998).

It remains to be determined whether the unique ability of DCs to prime naïve T cells results from the expression of molecules unique to DCs or from the high density of molecules involved in DC-T cell interactions. The number of MHC molecules and MHC/peptide complexes is 10- to 100-fold higher on DCs than on other APCs such as B cells and monocytes (Inaba *et al.*, 1997). Recognition of MHC-peptide complexes on DCs by antigen-specific T cell receptors (TCRs) constitutes "**signal 1**" in DC-T cell interactions. Subsequent DC-T cell clustering is mediated by several adhesion molecules. The Ig superfamily members ICAM-1 (CD54), ICAM-2 (CD50) and ICAM-3 (CD102), which are all ligands for the β_2 integrin, leukocyte function antigen-1 (LFA-1, CD11a/CD18), are expressed on DCs, but show differential regulation. ICAM-1 is expressed at low density on blood DCs and LCs but is quickly upregulated upon activation, whereas ICAM-2 and ICAM-3 showed little change in expression level with activation (Starling *et al.*, 1995). The recently identified lectin DC-SIGN has been shown to bind ICAMs expressed on T lymphocytes and on endothelia and is important for stabilising DC interaction with T cells (Geijtenbeek *et al.*, 2000).

The crucial factor, that constitutes "**signal 2**", required to sustain T cell activation, is the interaction between costimulatory molecules expressed by DCs and their receptors/ligands

expressed by T cells. B7.2/CD86 on DCs is so far the most critical molecule for amplification of T cell responses (Caux *et al.*, 1994). Although both B7.1/CD80 and B7.2 deliver a strong costimulatory signal via CD28 on T lymphocytes, blocking of B7.2 inhibits the MLR to a greater extent than blocking B7.1 (McLellan *et al.*, 1995) and B7.2 blockade reduces DC primary antigen specific responses (Fagnoni *et al.*, 1995).

T cells can activate DCs via CD40L-CD40 signalling, which leads to increased expression of B7.1/B7.2 and cytokine release (IL-1, TNFα, chemokines, and IL-12) (Bennett *et al.*, 1998; Cella *et al.*, 1996; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998). Triggering of CD40 on DCs results in upregulation of OX40 ligand (OX40L), which then signals naïve T cells to express IL-4 (Brocker *et al.*, 1999; Flynn *et al.*, 1998). Mature DCs also express 4-1BB ligand (De Benedette *et al.*, 1997). 4-1BB is a costimulator expressed primarily on activated $CD4^+$ and $CD8^+$ T cells. 4-1BB costimulation preferentially induces $CD8^+$ T cell proliferation and production of IFNγ (but not of IL-4) (Kim *et al.*, 1998). Engagement of RANK (receptor activator of NF-κB), a member of the TNF receptor family, by its ligand TRANCE (TNF-related activation induced cytokine) expressed on activated T cells, stimulates the secretion of cytokines such as IL-1, IL6, and IL-12 by DCs. This results in increased DC survival, by inhibition of DC apoptosis and, in turn, in enhanced proliferative T cell responses in mixed lymphocyte reactions (Josien *et al.*, 1999; Wong *et al.*, 1997).

I.2.2 DC polarisation

Differentiation of naïve $CD4^+$ T cells into IFNγ-producing effector T_H1 cells or IL-4, IL-5 and IL-13-producing effector T_H2 cells depends on the antigen, cytokines and the molecular microenvironment (reviewed in Murphy *et al.*, 2000). Accumulating data suggest that migrating DCs not only carry antigenic and costimulatory signals, but also are well equipped to transmit an additional "**signal 3**" from the periphery to the lymph nodes. Signal 3 is less defined than signal 1 and 2, but it is thought to contribute to the polarisation of naïve T_H cells towards T_H1 or T_H2, or to the induction of tolerance as discussed later (see section I.2.3). Possible candidates able to mediate signal 3 in the polarisation process are summarised in figure I.2.

In humans, monocyte-derived DCs (DC1) polarise naïve T cells predominantly towards a T_H1 profile, whereas the plasmacytoid DC subset (DC2) induces T cells to predominantly produce T_H2 cytokines (Rissoan *et al.*, 1999). However, myeloid DCs can give rise to either DC1 or DC2, depending on the nature of the maturation stimulus influencing IL-12 production (Kalinski *et al.*, 1999). IL-12, the critical T_H1 polarising cytokine (Trinchieri, 1995), is produced by DCs after stimulation with LPS, unmethylated bacterial CpG–containing DNA, double-stranded viral RNA, CD40 activation and IFNγ signalling (Cella *et al.*, 1999; Cella *et al.*, 1996; Hartmann *et al.*, 1999; Rescigno *et al.*, 1999), but is not produced in response to TNFα, IL-1, fungal hyphae and nematode products (Cella *et al.*, 1996; d'Ostiani *et al.*, 2000; Whelan *et al.*, 2000). Anti-inflammatory molecules such as IL-10, TGF-β, prostaglandin E_2 (PGE_2) and corticosteroids (Kalinski *et al.*, 1997; Piemonti *et al.*, 1999; Steinbrink *et al.*, 1997; Strobl and Knapp, 1999) inhibit DC maturation and IL-12 production, thereby inducing T_H2 or regulatory T (T_{reg}) cells (see section I.2.3). Skewing is not restricted to $CD4^+$ T cells, but also applies to $CD8^+$ T lymphocytes and NK T cells (Croft *et al.*, 1994; Kadowaki *et al.*, 2001; Morelli *et al.*, 2000).

In contrast to human plasmacytoid DCs which primarily induce T_H2 responses, murine splenic $CD8\alpha^+$ lymphoid DCs prime naïve $CD4^+$ T cells to make T_H1 cytokines, whereas splenic $CD8\alpha^-$ myeloid DC subsets and DCs isolated from Peyer's patches or the respiratory tract elicit a T_H2 response (Table I.1; Iwasaki and Kelsall, 1999; Maldonado-López *et al.*, 1999; Pulendran *et al.*, 1999; Stumbles *et al.*, 1998). DCs from IL-12-deficient mice fail to induce T_H1 responses, confirming a critical role for IL-12 in T_H1 responses induced by DCs (Maldonado-López *et al.*, 1999). A similar T_H1-deficient phenotype can be found in mice lacking IL-18. However, IL-18 appears to act mainly as a factor supporting ongoing T_H1 responses, rather than inducing T_H1 differentiation in the naïve T_H-cell population (reviewed in O'Garra, 1998). Lymphoid but not myeloid DCs can be induced to make large amounts of IL-12 and IFNγ (Maldonado-López *et al.*, 1999; Ohteki *et al.*, 1999). The mechanism by which myeloid DCs induce T_H2 cytokines is not clear, although IL-13 (McKenzie *et al.*, 1998), IL-6 (Rincon *et al.*, 1997a), and OX40L are good candidates (Flynn *et al.*, 1998). Interestingly, a recent report suggests a crucial role for CD40 in inducing a T_H2 response *in vivo* (MacDonald *et al.*, 2002), even though CD40 signalling is known to induce IL-12 production in DCs (see I.2.1). The involvement of B7.1 and B7.2 in T_H1/T_H2 polarisation remains unclear, but, in

some experimental systems, B7.1 was shown to promote T_H1 responses, whereas B7.2 ligation tended to skew toward T_H2 responses (Freeman *et al.*, 1995; Kuchroo *et al.*, 1995).

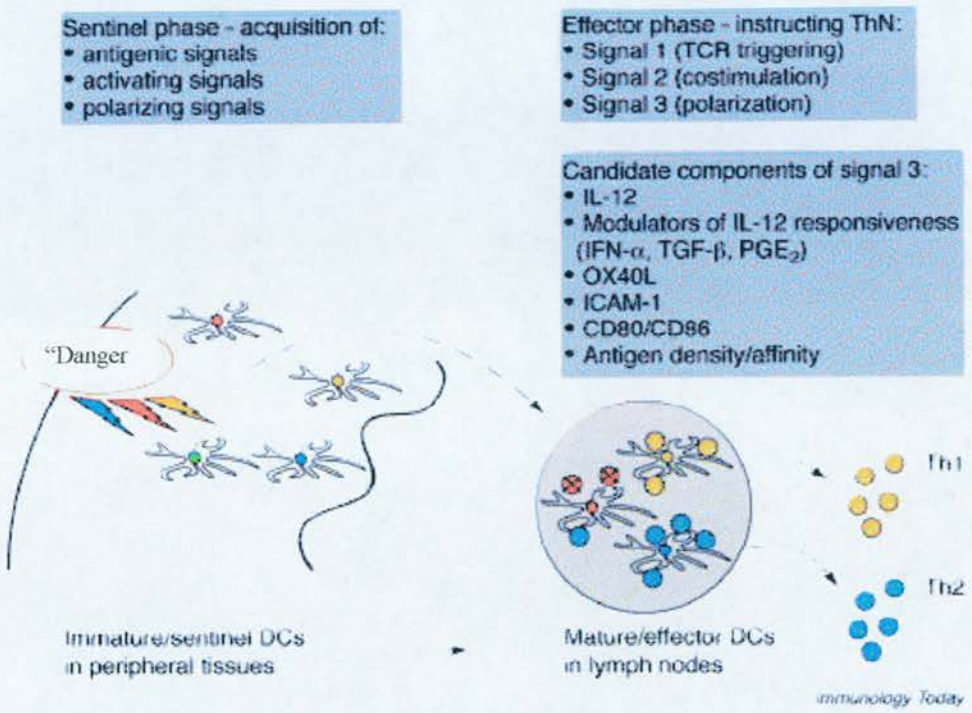


Figure I.2. A model showing the stages of learning and teaching in the life cycle of DCs. Activation of immature DCs in peripheral non-lymphoid tissues instructs them to take up antigen and migrate to the lymph nodes. Apart from collecting information about the antigenic structure of the pathogen, immature DCs learn about its pathogenicity, inferred from its DC-activating properties. At the same stage, DCs are susceptible to pathogen-derived or pathogen-induced factors that modulate their ability to produce IL-12. The ability of DCs to take up antigen and their susceptibility to functional modulation is either lost or is strongly reduced in fully mature DCs. This might help to restrict the acquisition of antigenic and polarising signals to the relevant site of pathogen entry, thereby limiting the risk of acquiring irrelevant signals at later stages. Within the lymph nodes, polarised mature DCs provide naïve T_H cells with: an Ag-specific "signal 1"; a "signal 2", which informs about the Ag -related pathogenic potential; and a "signal 3", which is the DC-dependent component of the initial polarisation of naïve T_H cells to a preferential production of T_H1- or T_H2-type cytokines. Reprinted from Kalinski *et al.* (1999) with permission from Elsevier Science.

Recently, a new concept for the generation of T_H2-polarising DCs was introduced (Fig. I.3). In response to LPS, DCs produce IL-12 only transiently (Langenkamp *et al.*, 2000). Consequently, soon after stimulation DCs prime a T_H1 response, whereas at later time points the same cells preferentially prime T_H2 and non-polarised T cells. Thus, the subsets, the differentiation and the maturation state of the DCs influence the polarisation of the T cell response. Overall, distinct DC subsets exist in mice and humans that differentially skew T_H responses depending on their maturation state.

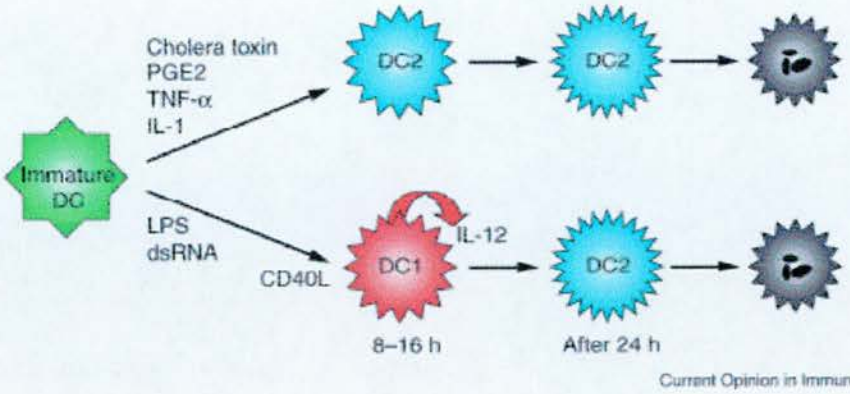


Figure I.3. Plasticity and kinetics of DC activation.
Some maturation stimuli fail to induce or inhibit IL-12 production (upper part). Consequently the mature DCs that are elicited (DC2) induce T_H2 responses, which are dependent on IL-4 produced by the responding T cells. Most pathogens or their products (e.g. LPS or double-stranded RNA), as well as T cells (via CD40L), stimulate IL-12 production (lower part). IL-12 is produced by DCs within a narrow time-window (8-16 hours), so that only recently stimulated DCs can induce T_H1 responses (DC1) whereas at later time-points (after 24h) the same DCs induce T_H2 responses. Nonviable DCs are shown in grey. Reprinted from Lanzavecchia & Sallusto (2001b) with permission from Elsevier Science.

I.2.3 DCs and tolerance

Evidence for a role of DCs in maintenance of peripheral tolerance

Tissue-specific DC types

In vitro, liver-derived DCs induce only weak proliferative or cytotoxic responses in allogeneic T cells compared with mature, bone marrow-derived DCs (Lu *et al.*, 1994). The liver appears to provide a microenvironment that might be important in modulating the function of DCs. Interestingly, hepatocytes and other liver cell types produce TGF-β. This cytokine has been implicated in the immunological privilege of the eye and is believed to induce tolerogenic APCs that migrate from the anterior chamber of the eye to regional lymphoid tissue, thus mediating systemic antigen-specific tolerance (Wilbanks and Streilein, 1992). TGF-β permits growth but suppresses the maturation of GM-CSF stimulated DCs (Yamaguchi *et al.*, 1997). If these TGF-β-treated DCs are injected prior to the transplantation, they prolong cardiac allografts survival in an antigen-specific manner (Lu *et al.*, 1997). Furthermore, TGF-β

increases production of IL-10 by hepatocytes (Ishizaka *et al.*, 1996). The effects of IL-10 on DCs will be discussed in more detail later. Recently, a novel population of mouse liver-derived DCs, propagated in response to IL-3 and CD40 ligation has been described (Lu *et al.*, 2001). These DCs appear to induce T_{reg} *in vitro* and prolong allograft survival. Taken together, these data indicate that immature liver-derived DCs may regulate peripheral immune responses by the induction of a T_{reg} cell population (Thomson and Lu, 1999; Thomson and Takayama, 1999).

The mucosal immune system has a unique immunological milieu that is based on two tolerance-inducing cytokines, IL-10 and TGF-β, and the milieu acts, in part, *via* the DCs to induce different phenotypes of T_{reg} cells. Pulmonary DCs that were isolated after respiratory exposure to antigen produced IL-10 and induced IL-10-expressing $CD4^+$ T_{reg} cells, whereas DCs isolated from the gut produced TGF-β upon oral antigen administration and induced TGF-β expressing $CD4^+$ T_{reg} cells (Akbari *et al.*, 2001).

Thymic DCs are capable of mediating negative, but not positive selection of thymocytes and therefore play a pivotal role in the induction of central tolerance (Brocker *et al.*, 1997). However, it is beyond the scope of this thesis to discuss the involvement of DCs in mediating central tolerance.

DC subsets

In vitro work suggests that in mice, both lymphoid and myeloid DCs can stimulate T cells, but that the lymphoid DC subset can limit the proliferation of T cells (Kronin *et al.*, 1996; Kronin *et al.*, 2000; Suss and Shortman, 1996). The lymphoid DCs appear to kill a proportion of the activated $CD4^+$ T cells in a Fas ligand dependent manner (Suss and Shortman, 1996), whereas they limit cytokine production of $CD8^+$ T cells (Kronin *et al.*, 1996). However, these effects were debated by others (McLellan and Kampgen, 2000). Lymphoid DCs seem to be unable to process complex protein antigens. When loaded with the appropriate peptide, however, these cells will stimulate potent T cell responses. Their inability to process protein antigens, together with the discovery that lymphoid DC in the T cell areas express high amounts of MHC class II/self-peptide complexes (Inaba *et al.*, 1997) makes lymphoid DCs ideal candidate APCs for tolerance induction (de St Groth, 1998; Inaba *et al.*, 1998). It is proposed that lymphoid DCs in the T cell area of LNs present antigen to T cells in a tolerogenic fashion, before the immunostimulatory myeloid DC (placed outside of the T cell area at the marginal zone) migrate into the T cell area and present antigen. Adjuvants, such as LPS, stimulate the

migration of the myeloid DC into the T cell area where they present their antigens in a stimulatory fashion, perhaps dominating over tolerance induction by the lymphoid-derived DC subset (Inaba *et al.*, 1998).

DC maturity

Recent data suggest a role for immature DCs in mediating tolerance presumably by the induction of T_{reg} cells (Fairchild and Waldmann, 2000). Repetitive stimulation of naïve $CD4^+$ T cells with allogeneic immature DCs *in vitro* resulted in alloreactive T_{reg} cells that produced high amounts of IL-10 and proliferated poorly after stimulation (Jonuleit *et al.*, 2000). The IL-10-producing T_{reg} cells can act directly on activated T_H1 cells and inhibit their antigen-specific proliferation and cytokine production in a cell contact-dependent manner. The suppressive activity of these T_{reg} cells, in contrast to the anergic T cells induced by IL-10 modulated DCs (see below), is non-specific and can be partially inhibited by the addition of exogenous IL-2 (Jonuleit *et al.*, 2000). Therefore, the functional activities of these T_{reg} cells induced by DCs *in vitro* are very similar to the described properties of the thymus-derived murine and human $CD4^+CD25^+$ T_{reg} cells *ex vivo* (Jonuleit *et al.*, 2001; Takahashi *et al.*, 2000; Thornton and Shevach, 1998).

Immature DCs are highly adapted to phagocytose apoptotic bodies (Albert *et al.*, 1998a; Albert *et al.*, 1998b). However as discussed, these DCs require to be "licensed" for efficient induction of cytotoxic T cell responses. One concept is that the stimulation of T cells by immature DCs will lead to tolerance because they only transmit "signal 1" from interaction with the MHC-peptide complex, whereas mature DCs produce immunity because they transmit an additional "signal 2" from costimulatory molecules (Guerder and Matzinger, 1992; Kurts *et al.*, 1997). Albert and colleagues challenged this concept by favouring the idea that a special "third signal", as described for T cell polarisation, is required to produce $CD8^+$ T cell tolerance. They found that mature but quiescent DCs induced tolerance, whereas mature DCs activated via CD40 ligation induced immunity (Albert *et al.*, 2001). The activation of mature DCs was mediated by $CD4^+$ T cells probably via CD40 crosslinking. They proposed the "third signal" for the regulation of priming vs. tolerising, which is active at the DC-$CD4^+$ T cell interface. Another report recently demonstrated the requirement for mature rather than immature DCs to induce tolerance (Menges *et al.*, 2002). Repetitive injections of TNFα-matured DCs pulsed with antigen but not immature or LPS/anti-CD40-treated DCs prevented induction of experimental autoimmune encephalomyelitis (EAE) *in vivo*, which was partially dependent on IL-10 produced by $CD4^+$ T cells. In summary, although immature DCs may

have been described to induce tolerance, it seems likely that they require a degree of maturity to interact with T cell and therefore, they would better be referred to as 'modulated'.

Transplantation tolerance

The first important clue that certain subtypes of DC are involved in peripheral tolerance induction *in vivo* stems from liver transplant studies (Calne *et al.*, 1969; Kamada *et al.*, 1981). The APCs that are thought to mediate the immune privilege of liver allografts are immature DCs. Donor interstitial DCs migrate to host secondary lymphoid tissue following organ transplantation, where they interact with specific, donor-reactive T cells (Thomson *et al.*, 1995). Immature myeloid-derived DCs propagated from normal murine liver are deficient in costimulatory molecules, although their expression of MHC and costimulatory molecules can be upregulated in response to appropriate factors *in vitro* (Lu *et al.*, 1994). They migrate *in vivo* to the T cell areas of secondary lymphoid tissue, where they persist for weeks in allogeneic recipients, a process called 'microchimerism' (Anderson and Matzinger, 2001). Consistently, it has been reported that depletion of donor APCs prevented the induction of transplantation tolerance (Josien *et al.*, 1998; Sun *et al.*, 1995). Furthermore, in some cases tolerance was re-established if donor-type DCs were cotransferred with APC-depleted grafts (Josien *et al.*, 1998). This suggests that DCs of the allografts, in addition to their proposed immunogenicity (Steinman, 1991), may also be important in establishing transplantation tolerance.

Strategies for DC modulation in inducing/maintaining peripheral tolerance

IL-10 modulated DCs

Originally, IL-10 was identified as a cytokine-synthesis-inhibiting factor, particularly with regard to the suppression of IFNγ production by T_H1 cells (Moore *et al.*, 2001). Stimulation of T cells *in vitro* was significantly reduced in the presence of IL-10 (Groux *et al.*, 1996). However, this effect was dependent on the presence of APCs (Groux *et al.*, 1998). Subsequently, the effects of IL-10 on APCs and in particular on DCs were identified. The immunosuppressive properties of IL-10 on DCs are caused by a reduction in the upregulation of expression of MHC class II molecules and several costimulatory and adhesion molecules and, in the human system, also the DC-specific marker CD83 (Bellinghausen *et al.*, 2001;

Buelens *et al.*, 1995; Chang *et al.*, 1995; Sato *et al.*, 1999; Steinbrink *et al.*, 1997). IL-10 treated DCs did not produce inflammatory cytokines such IL-1β, IL-6 and TNFα or IL-12 (Bellinghausen *et al.*, 2001; Brossart *et al.*, 2000; Buelens *et al.*, 1997; Koch *et al.*, 1996; Takenaka *et al.*, 1997). However, the effect of IL-10 on DCs was only seen if IL-10 was added to immature DCs, indicating that IL-10 modulates the function of immature DCs and inhibits their terminal differentiation, whereas mature DCs are resistant to IL-10 (Steinbrink *et al.*, 1999; Steinbrink *et al.*, 1997). Furthermore, immature DCs produce low amounts of IL-10, which *in vitro* prevents spontaneous maturation (Corinti *et al.*, 2001). *In vivo*, IL-10 producing DCs have been shown to suppress EAE (Yang *et al.*, 2000) and mediate tolerance to intranasally administered antigens (Akbari *et al.*, 2001).

Human IL-10-treated DCs from peripheral blood induce an antigen- and alloantigen-specific anergy of $CD4^+$ and $CD8^+$ T cells *in vitro* (Steinbrink *et al.*, 1999; Steinbrink *et al.*, 1997). This state of anergy was characterised by an inhibited T cell proliferation and reduced production of IL-2 and IFNγ and lack of cytotoxic function by the $CD8^+$ T cells. The induction of anergy requires direct cell-cell contact between T cells and DCs as well as soluble factors produced by IL-10-treated DCs. In contrast to T_{reg} cells, anergic T cells induced by IL-10-treated DCs are characterised by a markedly reduced expression of CD25. Furthermore, these anergic T cells did not produce immunomodulatory cytokines such as IL-10 or TGF-β (Steinbrink *et al.*, 1999; Steinbrink *et al.*, 1997). Comparisons of the stimulatory capacity of human DCs isolated from responding or progressing melanoma metastases demonstrated that the latter induced a markedly decreased T cell proliferation in a MLR resulting in alloantigen-specific anergy. This is consistent with the high amount of IL-10 produced by melanoma cells of the progressing metastases (Enk *et al.*, 1997). As a consequence, this finding supports earlier data showing that the production of IL-10 by tumour cells and/or tumour-infiltrating lymphocytes might serve as a mechanism for tumour-induced anergy. The ultraviolet (UV) irradiation resulting in immunosuppression appears to be mediated by the induction of IL-10 in keratinocytes and macrophages (Elmets *et al.*, 1983; Kang *et al.*, 1994). After migration to the draining LNs, the UV or IL-10 modified Langerhans cells induce tolerance in T cells (Elmets *et al.*, 1983; Enk *et al.*, 1993).

Designer DCs

Genetically engineered DCs that for example express IL-10 (Takayama *et al.*, 1998), TGF-β (Lee *et al.*, 1998), FasL (Min *et al.*, 2000) or CTLA-4-Ig (O'Rourke *et al.*, 2000) can induce alloantigen-specific T cell hyporesponsiveness and enhance the survival of allografts. However, these approaches have not yet succeeded in establishing the long-term allograft survival that would be indicative of a more robust form of tolerance. The use of techniques such as serial analysis of gene expression (SAGE) or oligonucleotide microarrays and their application to populations of DCs have already begun to reveal novel differentially expressed genes whose overexpression or mutation would permit the generation of 'designer' DCs with previously unexplored properties (Granucci *et al.*, 2001; Hashimoto *et al.*, 1999).

I.3 T cell signalling and polarisation

Activation and function of T lymphocytes are tightly regulated by signal transduction pathways that include specific cell-surface receptors, intracellular signalling molecules and nuclear transcription factors. As mentioned earlier, T cells require two signals to become activated, "signal 1" being the TCR triggering and "signal 2" being costimulation. The "signal 3" leading to polarisation or tolerance of the T cells is less defined (Fig. I.2). Here I will give a brief overview about the signalling pathways downstream of the TCR and the development of T helper subsets, which are reviewed in more detail by (Acuto and Cantrell, 2000; Chambers, 2001; Kuo and Leiden, 1999; Murphy *et al.*, 2000).

I.3.1 Signal 1/TCR signalling pathway

The TCR is a heterodimer of an α and β chain and interacts with the MHC/peptide complex. The affinity, avidity and stability of the MHC/peptide-TCR interaction vary over a wide range. This explains the variable requirement for stabilisation by CD4 and CD8. These coreceptors present on $CD4^+$ and $CD8^+$ T cells, respectively, are important to deliver the protein tyrosine kinase (PTK) Lck, which is associated with the intracellular end of CD4 and CD8 (Fig. I.4; Kuo and Leiden, 1999).

The TCR is associated with the CD3 cluster, which is composed of subunits γ, δ, ε and the ζζ homodimer chains (Weiss, 1993). These chains display in their cytoplasmic tails motifs termed ITAMs (immuno-receptor tyrosine-based activation motifs). Upon engagement of the TCR by antigen presented on MHC molecules, the protein tyrosine kinases (PTKs) of the Src family (Lck and Fyn) are activated and phosphorylate ITAMs of the CD3 complex, which in turn promotes the recruitment and subsequent activation of another tyrosine kinase ZAP-70 (zeta/ζ associated protein of 70kD) (Fig. I.4; Chan *et al.*, 1992; Straus and Weiss, 1992; Zenner *et al.*, 1996). Upon activation ZAP-70 phosphorylates adapter proteins LAT (linker for activation of T cells) and SLP-76 (src homology 2 domain-containing leukocyte phosphoprotein of 76kD), which transduce signals further resulting in the activation of the Ras/MAPK pathway, calcium mobilisation and cytoskeletal reorganisation (Acuto and Cantrell, 2000; Pivniouk and Geha, 2000). These early events appear to be crucial for the triggering of the signalling cascades that stimulate nuclear transcription factors that regulate the production of several cytokines, including IL-2. IL-2 gene transcription, a key event in T cell activation and proliferation, is regulated by the coordinate action of multiple factors including NF-AT, AP-1 and nuclear factor κB (NF-κB) (Herndon *et al.*, 2001; Kuo and Leiden, 1999). Activation of these different transcription factors regulating IL-2 gene transcription results from at least two synergising transduction pathways triggered at the TCR, as well as from costimulatory pathways ("signal 2") discussed later.

The Calcium-Calcineurin pathway

One of these cascades involves phosphorylation and activation of phospholipase Cγ (PLCγ), which cleaves the membrane lipid phosphatidylinositol bisphosphate to generate diacylglycerol (DAG) and inositol trisphosphate (IP_3). These second messengers are essential for T cell activation. DAG activates protein kinase C (PKC), whereas IP_3 triggers an increase in the concentration of intracellular calcium (Fig. I.4; Weiss and Imboden, 1987). The increased Ca^{2+} level then activates the protein phosphatase Calcineurin by disrupting the inhibitory effects of Calmodulin. Calcineurin activation leads to the dephosphorylation of the nuclear factor of activated T cells (NF-AT), allowing it to enter the nucleus, where it cooperates with other transcription factors to bind to variety of promoters such as, for example, the IL-2 promoter (Rao *et al.*, 1997; Wulfing *et al.*, 1997). In *in vitro* studies, the effect of this necessary calcium signal for activation of gene transcription factors can be induced by the action of calcium ionophores such as ionomycin. In combination with phorbol esters, which stimulate PKC such as phorbol myristate acetate (PMA), ionomycin fully activates IL-2 gene transcription.

The MAPK Pathways

Mitogen activated protein kinases (MAPKs) are important in many signalling pathways, e.g. growth hormone, TNFα or IL-1 signalling, leading to cell growth and to stress responses. "Signal 1" activates several MAPKs: DAG and PKC activate Ras, which subsequently stimulate a number of serine/threonine kinases and dual-specificity kinases leading to the activation of the MAPKs ERK (extracellular signal receptor regulated kinase) (Robbins *et al.*, 1994), JNK (Jun N terminal kinase) and p38. These MAPKs directly phosphorylate transcription factors involved in the formation of the heterodimeric transcription factor AP-1 (Jun/Fos).

Another transcription factor important for the generation of IL-2 is NF-κB. Activation of NF-κB is dependent on stimulation of the TCR and costimulation via CD28. The serine/threonine kinase Akt and the MAPKs participate in the induction of IκB degradation. Free from its association with IκB, NF-κB can move into the nucleus and activate transcription (Kane *et al.*, 2001).

Many recent studies have focused on functional and physical interactions between the TCR and cytoskeleton such as TCR clustering at the site of APC contact and reorientation of the microtubule-organising centre. Aggregation of several TCRs on the T cell surface promotes concentration of lipid- and kinase-rich "rafts" or "microdomains" thereby facilitating signalling (Acuto and Cantrell, 2000; Viola *et al.*, 1999).

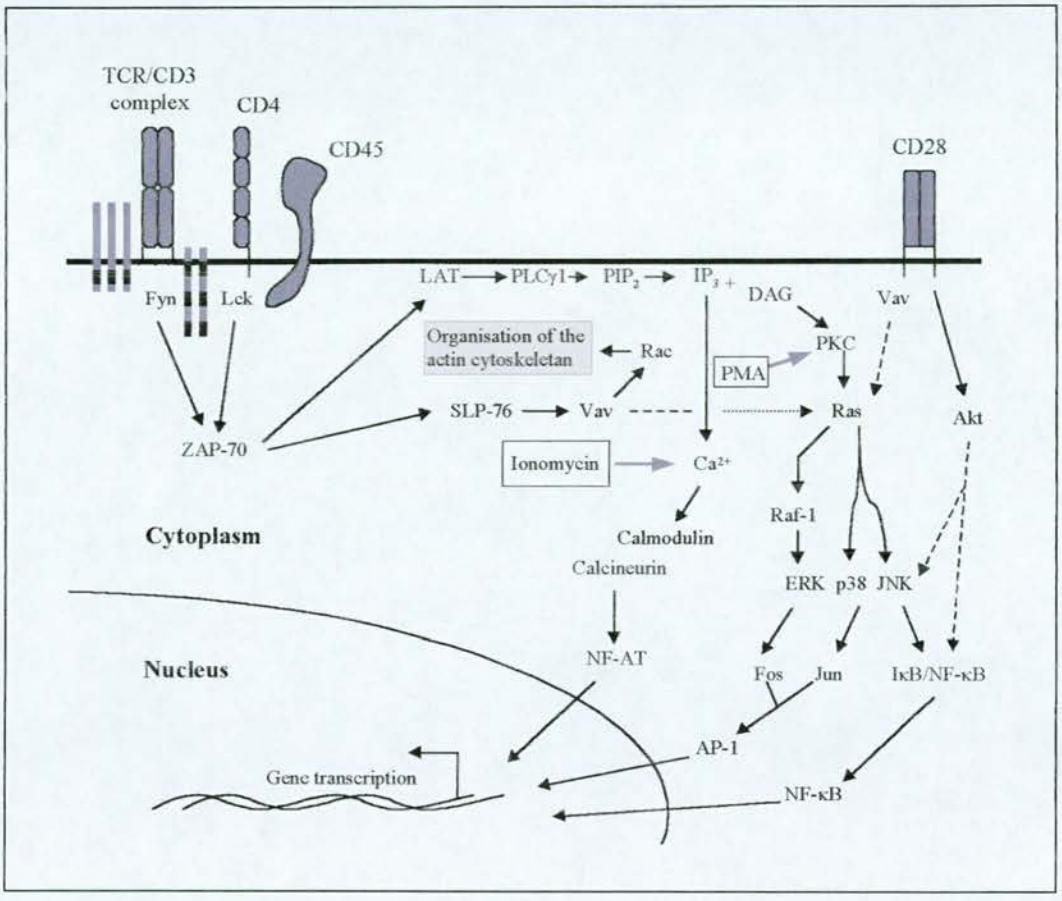


Figure I.4. T cell receptor signalling.
The TCR/CD3 complex is a substrate for the Src family of protein tyrosine kinases (PTKs) Lck and Fyn, which phosphorylate tyrosine residues on the intracellular tail of the subunits. These sites, known as immuno-receptor tyrosine-based activation motifs (ITAMs, black boxes), are the docking sites for proteins with Src homology 2 (SH2) domains. One such protein is the PTK ZAP-70. Initial activation of PTKs is followed by phosphorylation activation of downstream components as delineated in the figure. These events ultimately lead to regulation of gene expression by the activation of transcription factors such as NF-AT, AP-1 and NK-κB. Arrows indicate activation of the target molecule. This activation can be direct or involve other proteins. Dashed arrows: Direct biochemical evidence for this pathway is still missing. Targets of mitogenic stimuli PMA/ionomycin are indicated by bold grey arrows.

I.3.2 Signal 2/CD28 cosignalling pathway

In addition to the TCR/CD3 associated signalling cascade, a costimulatory "signal 2" is required for full T cell activation and proliferation (Chambers, 2001). TCR ligation without costimulation can result in functional inactivation and anergy and in cell death (Boise *et al.*, 1995; Gimmi *et al.*, 1993; Harding *et al.*, 1992). The best-characterised costimulatory receptor expressed on a resting T cell is CD28. CD28 is a homodimeric glycoprotein expressed on 95% of $CD4^+$ T cells and on approximately 50% of $CD8^+$ T lymphocytes (June *et al.*, 1994). Upon interaction with its ligands B7.1 and B7.2 expressed on the APC, CD28 enhances IL-2 synthesis and T cell proliferation (reviewed in Chambers, 2001). Evidence has been found showing that the role of CD28 signalling is not to costimulate the initial activation of naïve T cells but to sustain the late proliferative response and enhance long-term cell survival (Sperling *et al.*, 1996).

CD28 ligation triggers PLCγ activation and Ca^{2+} mobilisation (Ledbetter and Linsley, 1992). Although CD28 does not display any obvious intrinsic enzymatic activity, tyrosine residues on its cytoplasmic tail can be phosphorylated, allowing the binding of different signal transducing molecules. The signalling cascade that bridges these early CD28-triggered events with the later activation of nuclear factors regulating cytokine gene transcription is still unresolved. The guanine exchange factor Vav associated with activated CD28 may link TCR and CD28 stimulation by activating the Ras/MAPK pathway (Fig. I.4; Klasen *et al.*, 1998; Villalba *et al.*, 2000). Further it was suggested that NF-κB and AP-1 are direct targets for CD28 signals (Edmead *et al.*, 1996), the latter via JNK signalling. It is noteworthy that activation of JNK in T cells requires ligation of both TCR and CD28 (Su *et al.*, 1994). This result led to the conclusion that JNK activation could be a nodal event between the TCR-induced signalling pathway and the CD28-mediated costimulation pathway, resulting in IL-2 production. CD28 signalling also stabilises IL-2 mRNA post-transcriptionally by an unknown mechanism (Lindsten *et al.*, 1989), which is an important mechanism of costimulation (Umlauf *et al.*, 1995).

When CD28 and TCR are both triggered, the T cell does not proliferate indefinitely. The membrane-spanning tyrosine phosphatase CD45, which plays an essential role in T cell activation, is also involved in the termination of the T cell response via dephosphorylation of the CD3ζ chain (Furukawa *et al.*, 1994). Furthermore, the engagement of TCR activates the

expression of CTLA-4, a cell surface molecule structurally related to CD28 (reviewed in Chambers *et al.*, 2001). Like CD28, CTLA-4 also recognises B7.1 and B7.2. However, CTLA-4 provides a crucial and unique negative signal that inhibits IL-2 secretion (Blair *et al.*, 1998). CTLA-4 is a critical negative regulator, which means that "signal 1" and "signal 2" will result in the T cell eventually being turned off under physiological conditions.

Another mechanism to limit TCR signalling is its downregulation, which is an inhibitory feedback regulation characteristic of many receptor systems. Internalisation of the TCR from the cell surface begins a few minutes after initiation of TCR signalling and is followed by degradation (Valitutti *et al.*, 1997). Additionally, there exist biochemical interference of TCR signalling by downstream molecules such as c-Cbl or SHP-1 (reviewed in Germain and Stefanova, 1999).

I.3.3 Polarisation from the view of T cells

T_H1 and T_H2 subsets were originally defined by Mosmann & Coffman (Mosmann *et al.*, 1986). T_H1 cells secrete the cytokines IL-2 and IFNγ, activate phagocytes and promote protection against intracellular pathogens. T_H2 cells secrete the cytokines IL-4, IL-5, IL-10 and IL-13, which affect humoral immunity to extracellular parasites and are responsible for immune responses to persistent antigens, for instance allergens (reviewed in Constant and Bottomly, 1997). The two T helper subsets also cross-regulate each other, so the balance between T_H1 and T_H2 cytokines can determine whether the immune response is appropriate for the host or may result in the generation of pathological responses. Overproduction of T_H1 cytokines has been implicated in delayed-type hypersensitivity reactions and autoimmune diseases. T_H2 cytokines recruit eosinophils and activate mast cells; thus dysregulation of T_H2 cytokines can lead to allergic and inflammatory conditions. $CD8^+$ T cells can also be polarised to effector subsets with distinct cytokine production profiles similar to those found in $CD4^+$ T cells (Croft *et al.*, 1994). This polarisation has been observed both *in vitro* and *in vivo* (Morelli *et al.*, 2000; Sad *et al.*, 1995), and it is likely that $CD8^+$ T cells also play an important regulatory role during immune responses.

The TCR signalling, the nature of costimulation and the cytokine milieu influence the polarisation of T cells (see also section I.2.2). IL-12 promotes IFNγ production and T_H1 development via signalling pathways that lead to activation of STAT4 (signal transducer and activator of transcription 4) (Murphy *et al.*, 2000). Ligation of the IL-4 receptor by IL-4, leading to STAT6 activation, can drive a naïve T_H cell down a T_H2 differentiation pathway. Thus, cytokines have emerged as critical inducers of T_H subset development (Fig. I.5). Overall, the mutually antagonistic effects of IL-4 and IFNγ regulate T_H1/T_H2 balance and subsequent polarisation. Understanding the transcriptional regulation of IFNγ as a hallmark cytokine for T_H1 and IL-4 for T_H2 development is therefore critical for elucidating the process of T_H cell differentiation.

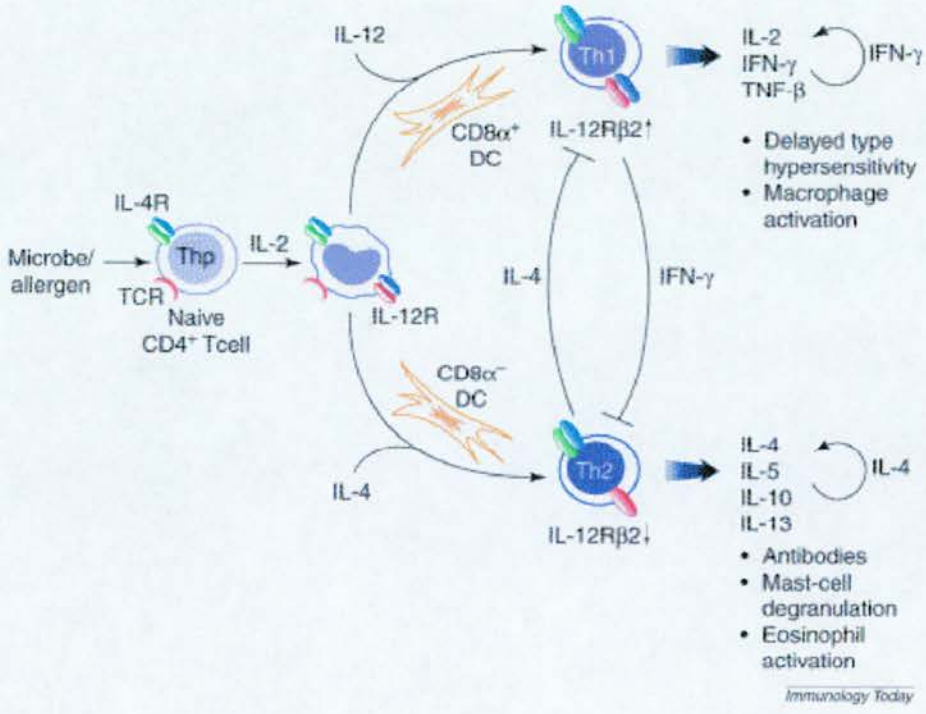


Figure I.5. Overview of T_H cell differentiation.
A naïve $CD4^+$ T cell is activated via the TCR when it encounters antigen presented by an APC. Once activated, the T_H cell starts to proliferate, secrete IL-2, and express the IL-12 receptor consisting of a β_1 and a β_2 chain. On encountering IL-12 secreted by macrophages and/or DCs, a T_H1 differentiation programme is initiated. The expression of IL-12Rβ_2 chain increases in the developing T_H1 cell and ligation of the IL-12R by IL-12 leads to the activation of STAT4 and initiation of the T_H1 differentiation program. IL-4 produced by several cell types namely other T_H2 cells, mast cells, basophils and eosinophils induces differentiation into the T_H2 subset and downregulation of IL-12Rβ_2 expression. Reprinted from Rengarajan *et al.* (2000) with permission from Elsevier Science.

I.3.3.1 T_H1 development

Although STAT4 is expressed by T_H1 and T_H2 cells (Szabo *et al.*, 1995), STAT4 activation by IL-12 occurs only in T_H1 cells because of the T_H1-restricted expression of the IL-12Rβ_2 subunit (Fig. I.6; Szabo *et al.*, 1997). Even though STAT4 activation was correlated with the capacity to promote IFNγ production (Szabo *et al.*, 1995), not all IFNγ production appears to depend on STAT4 (Kaplan *et al.*, 1998). $CD8^+$ T cells, for example, exhibit STAT4-independent TCR-induced IFNγ production (Carter and Murphy, 1999).

Members of the interferon regulatory factor (IRF)-1 family have been suggested to participate in T_H1 development and IFNγ production. IL-12 signalling induces IRF-1 expression in T cells through STAT4 activation, which is required for IL-12-induced IFNγ production (Coccia *et al.*, 1999; Lohoff *et al.*, 1997). Thus, IRF-1 expression may be downstream of IL-12, but direct actions in the IFNγ promoter are not clear. Another protein induced by IL-12 via STAT4 in T_H1 is ERM, an Ets family transcription factor (Ouyang *et al.*, 1999). However, ERM expression did not restore IFNγ transcription in STAT4-deficient T cells. T-bet (T-box expressed in T cells), a member of the T-box family of transcription factors that regulate several developmental processes, was identified as T_H1-specific (Szabo *et al.*, 2000). T-bet strongly activates IFNγ production when expressed in naïve and type 1 or type 2 polarised $CD4^+$ and $CD8^+$ T cells and simultaneously represses the T_H2 cytokines IL-4 and IL-5 (Szabo *et al.*, 2000). Finally, the homeodomain transcription factor Hlx was found to be T_H1-specific, with transgenic overexpression enhancing T_H1-type responses (Flavell *et al.*, 1999; Murphy *et al.*, 2000).

While TCR signalling was considered a required stimulus for IFNγ production by $CD4^+$ T cells, recently Robert *et al.* discovered a second pathway for induction. The cytokines IL-12 and IL-18 together induced full IFNγ production by T_H1 cells, independent of stimulus through the TCR (Robinson *et al.*, 1997). IL-18 receptor is a new member of the IL-1 receptor family and its signalling pathway shares several components with the IL-1 and TLR pathway (reviewed in Akira *et al.*, 2001). Molecular mechanisms of IL-18R signalling in T_H1 cells involves recruitment of IL-1R associated kinase (IRAK) and MyD88 which induce NF-κB activation through TNF receptor associated factor 6 (TRAF6) (Adachi *et al.*, 1998; Robinson *et al.*, 1997).

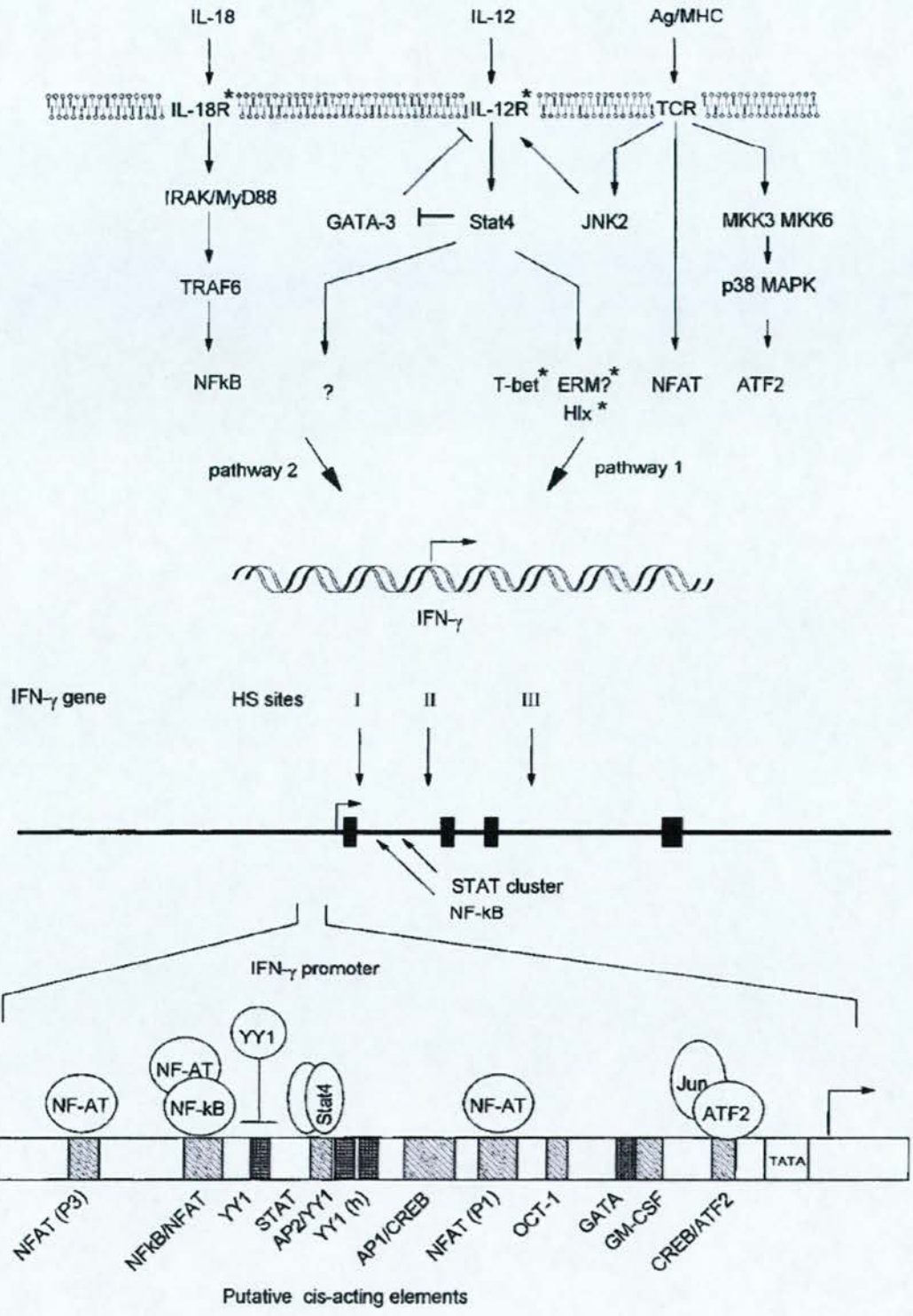


Figure I.6. Pathways and transcription in IFNγ production by T_H1 cells.
T_H1-specific components (asterisks) include the receptors for IL-12 and IL-18 and recently identified transcription factors. A number of putative *cis*-acting elements are shown, but none are known to interact with T_H1-specific transcription factors. STAT4 activation is restricted to T_H1 cells, but alone is not sufficient to transactivate the IFNγ gene. HS sites: DNase I hypersensitivity sites. Reprinted from Murphy *et al.* with permission from the Annual Review of Immunology, Volume 18, © 2000, by Annual Reviews www.AnnualReviews.org.

The stability of the T_H1 phenotype is not clear, but recently IL-4 signalling impairment was reported for T_H1 cells (Huang and Paul, 1998). Diminished IL-4 signalling in T_H1 cells may involve suppressor of cytokine signalling (SOCS) proteins (Losman *et al.*, 1999). SOCS family members modulate signalling by several mechanisms, which include inactivation of the Janus kinases (JAKs), blocking access of the STATs to receptor binding sites and ubiquitination of signalling proteins and their subsequent targeting to the proteasome (reviewed in Krebs and Hilton, 2001).

I.3.3.2 T_H2 development

Following priming with IL-4, T_H2 populations become progressively less susceptible to reversal by T_H1-inducing cytokines, and IL-4 production becomes independent of extrinsic IL-4 (Huang *et al.*, 1997; Murphy *et al.*, 1996). Loss of IL-12R expression by T_H2 cells may explain early T_H2 stability (Szabo *et al.*, 1995).

IL-4R signalling involves the IL-4-specific α chain and common gamma chain (γc). Engagement of IL-4R leads to activation of STAT6 by Janus kinases, Jak1 and Jak3 (Fig. I.7; reviewed in Nelms *et al.*, 1999). The expression of transcription factor GATA-3, which is selectively expressed in T_H2 cells, is induced in a STAT6-dependent manner and increases expression of T_H2 cytokines (Chtanova *et al.*, 2001; Ouyang *et al.*, 1998; Zheng and Flavell, 1997). Additionally, it blocks IL-12R expression and prevents T_H1 development independently of the induction of IL-4 (Ouyang *et al.*, 1998). Further, the T_H2-specific transcription factor c-Maf also attenuates T_H1 differentiation by both IL-4-dependent and independent mechanisms in addition to directly augmenting IL-4 promoter activity through cooperative interactions with NF-AT and JunB (Ho *et al.*, 1996; Ho *et al.*, 1998; Rincon *et al.*, 1997b).

Several factors are known to inhibit IL-4-induced transcription of type 2 cytokines. As previously mentioned, SOCS-1 is likely to inhibit Jak1 and Stat6 activation in response to IL-4 signalling in T_H1 cells (Losman *et al.*, 1999). Bcl-6 was found to repress T_H2 cytokines by binding to a transcription site recognised by STAT6 (Dent *et al.*, 1997).

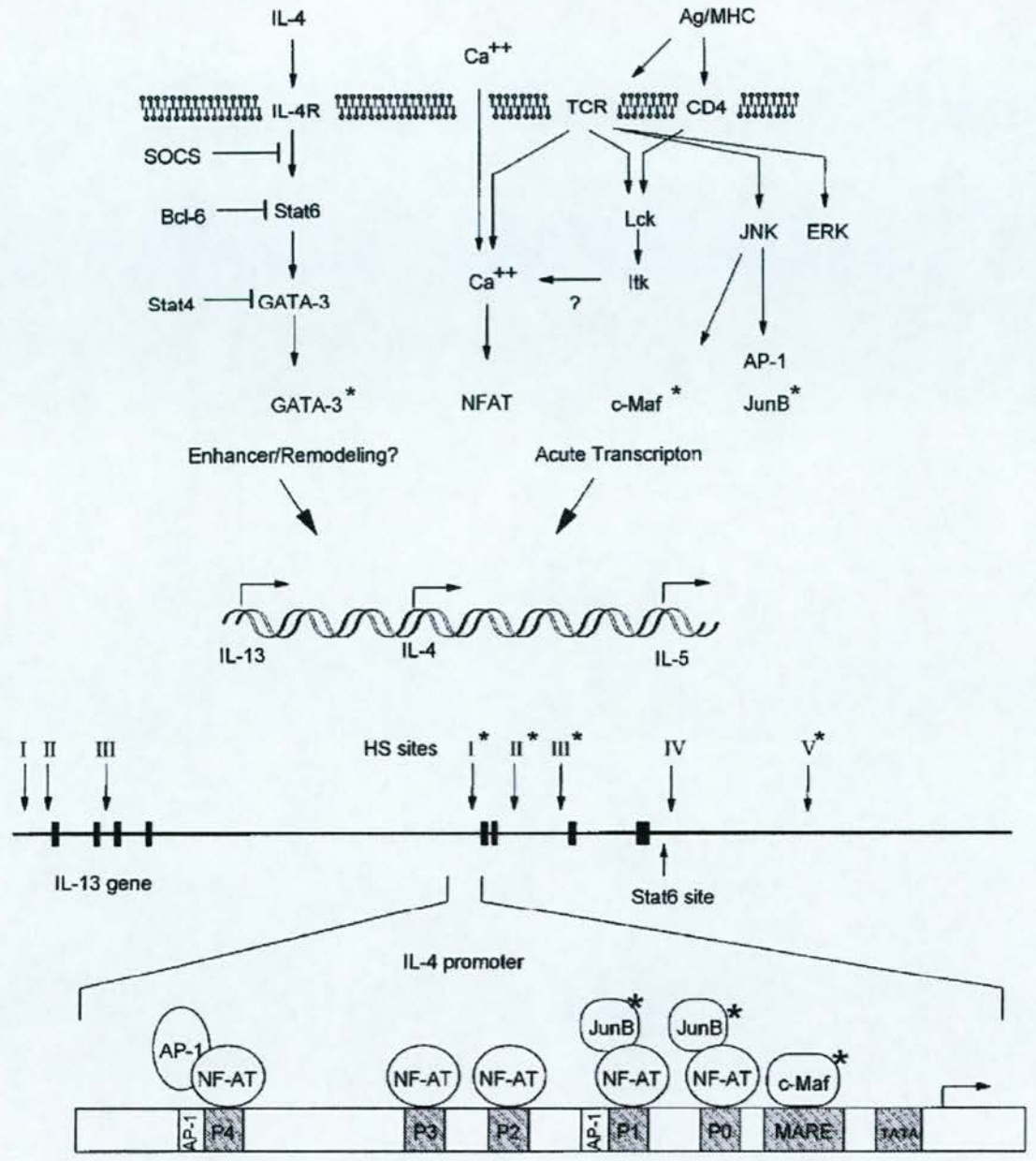


Figure I.7. Pathways and transcription in IL-4 production by T_H2 cells.
T_H2-specific components (asterisks) include the transcription factors GATA-3, c-Maf, and JunB. Acute transcriptional activation by NF-AT, c-Maf, JunB, and perhaps other factors may require prior chromatin remodelling of the IL-4 locus or enhancer activity provided by GATA-3. T_H2-specific DNase-hypersensitive (HS) sites are indicated with asterisks. Reprinted from Murphy *et al.* with permission from the Annual Review of Immunology, Volume 18, © 2000, by Annual Reviews www.AnnualReviews.org.

After primary activation, cytokines are expressed for several days. Recent studies have linked cell division with the acquisition of effector cytokine expression (Bird *et al.*, 1998; Gett and Hodgkin, 1998; Richter *et al.*, 1999). Although T cells were immediately capable of making IL-2, production of IFNγ and IL-4 correlated with the number of cell divisions following activation. In particular, IFNγ appeared to require one to two cell divisions, and IL-4 production was delayed until four cell divisions (Bird *et al.*, 1998; Gett and Hodgkin, 1998). The expression of cytokines is "memorised" by T_H cells. When restimulated, they recall expression of those cytokines they had been instructed to express earlier, without requirement for the original costimulator (Murphy *et al.*, 1996; Szabo *et al.*, 1995). Recently, several groups have provided evidence for somatic imprinting of cytokine genes as a molecular basis for cytokine "memory" in T_H lymphocytes. Analysis of DNA methylation and DNase I hypersensitivity of the genomic regions of various cytokine genes has shown that those cytokine genes, which the T cells had originally expressed, remained "accessible" later, and that accessibility is correlated with the ability for expression of the cytokines upon recall stimulation (Agarwal and Rao, 1998; Bird *et al.*, 1998). Cells committed to the type 1 or type 2 phenotypes demonstrate changes in chromatin structure around IFNγ and IL-4, respectively. Such structural changes are believed to begin the process by which the cytokine expression repertoire is stabilised, such that effector functions become passed on to daughter cells.

I.4 The Notch Pathway

In many situations in development, the choice between alternative cell fates is controlled by cognate interactions between cells mediated via the transmembrane receptor Notch. Vertebrate Notch and its *Caenorhabditis* (*C.*) *elegans* homologues LIN-12 and GLP-1 comprise a family of receptors that participate in cell fate decisions throughout embryonic development. The basic signalling mechanism of the Notch family of receptors shows a striking evolutionary conservation. In spite of this conservation in mechanism, the Notch family of receptors are extraordinarily adaptable and have been co-opted to function in many diverse developmental processes.

I.4.1 Notch receptors and ligands

Human Notch was first identified as a gene involved in chromosomal translocation detected in some T-cell leukaemias. This gene, called TAN-1, was shown to generate an intracellular activated form of Notch1 (ICN1) (Ellisen *et al.*, 1991), and can induce T-cell leukaemias with high frequency when expressed in murine bone marrow cells (Capobianco *et al.*, 1997). To date, four mammalian Notch homologues (Notch1-4), which interact with transmembrane-bound ligands such as Jagged1, Jagged2, Delta-like1, Delta-like3 and Delta-like4 (Delta-like1, 3, 4 proteins are referred to as Delta1, 3, 4, respectively) have been identified (Table I.3; Fleming, 1998; Yoneya *et al.*, 2001). Notch receptors share a high degree of structural homology between members as well as across species as diverse as flies and humans.

The Notch extracellular domain contains a variable number (29-36) of tandem epidermal growth factor (EGF)-like repeats and three Lin/Notch repeats (LNR), which function in ligand binding and Notch activation (Fig. I.8; Lardelli *et al.*, 1994; Rebay *et al.*, 1993; Rebay *et al.*, 1991). The conserved cysteines between the LNR and the transmembrane domain (TM) are probably involved in disulphide bonding of the heterodimeric receptor. The intracellular domain of Notch receptors consists of a RAM domain, 6 ankyrin (ANK) repeats, 2 nuclear localisation sequences (NLS), a glutamine-rich domain (OPA), a transcriptional activation domain (TAD) and a C-terminal proline-glutamate-serine-threonine-rich (PEST) domain, which is thought to be a degradation signal (Rogers *et al.*, 1986). Notch4 is an exception in that it has a shorter intracellular domain that lacks one of the two NLS (Uyttendaele *et al.*, 1996). The RAM domain is the primary binding site for the CSL (CBF1, Su(H), LAG-1) effectors (Tamura *et al.*, 1995). However, the TAD and the ANK repeat domain can also interact with C promoter binding factor-1 (CBF1)/recombination signal binding protein Jκ (RBP-Jκ) (Aster *et al.*, 2000; Fortini and Artavanis-Tsakonas, 1994; Kurooka *et al.*, 1998). ANK repeats are characteristic motifs involved in protein-protein interactions; the ANK repeat domain is the most highly conserved region and is essential for Notch signal transduction. In addition to CBF1, the ANK domain also interacts with proteins such as Deltex and Mastermind, which modulate Notch signalling. Recently, a NF-κB binding domain (NBD) has been identified, which overlaps with a NLS and parts of the RAM domain (Wang *et al.*, 2001).

The two distinct but related classes of Notch ligands, Jagged/Serrate and Delta, encode transmembrane proteins, which contain 16 and 8 EGF repeats, respectively, on the extracellular side (Bettenhausen *et al.*, 1995; Luo *et al.*, 1997; Oda *et al.*, 1997; Shimizu *et al.*, 1999). In addition, they harbour a modified EGF repeat at the N-terminal region referred to as the DSL (Delta/Serrate/Lag-2) domain, which is probably involved in receptor binding. The principal difference between the two types of ligand is that Serrate-like ligands contain a cysteine-rich (CR) region, whereas Delta-like ligands do not. The intracellular domains of Delta and Serrate are short and show little structural conservation.

Table I.3. Conserved components of the Notch signalling pathway.

Organism	Notch receptors	Ligands *		Intracellular effectors	Target Genes
Drosophila	Notch	Serrate	Delta	Su(H)	E(spl)
C. elegans	Lin-12, Glp-1	Apx-1	Lag-2	Lag-1	
Vertebrates					
Human	Notch1-4	Jagged1, 2	Delta-like 1, 3, 4	CBF1/RBP-Jκ	Hes1, 5, 7
Mouse	Notch1-4	Jagged1, 2	Delta-like 1, 3, 4	CBF1/RBP-Jκ	Hes1, 5, 6, 7
Rat	Notch1-3	Jagged1	Delta-like 3		Hes1, 5
Xenopus	Xotch		X-Delta1, 2	XSu(H)1-2	XSu(H)1-2
Zebrafish	Notch1-3, 5, 6	SerrateB	Delta A, B, D		

* It is still unclear to what extent different ligands function to activate specific receptors under physiological conditions

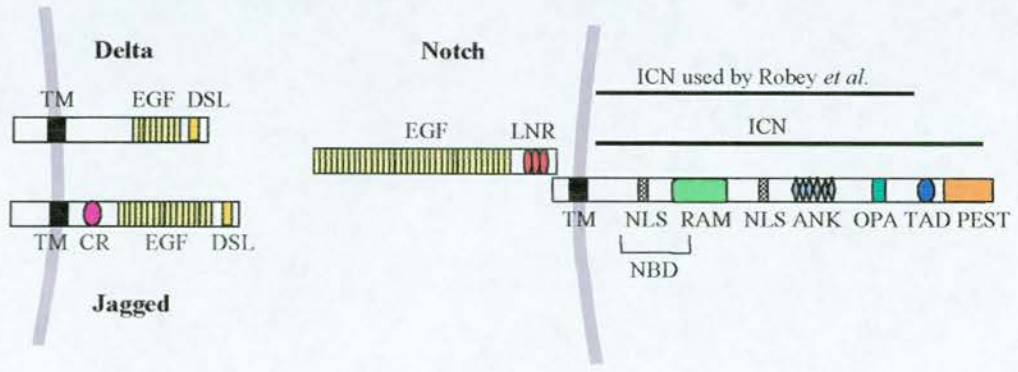


Figure I.8. Schematic structure of Notch and the ligands Jagged and Delta.
ANK, 6 ankyrin repeats; CR, cysteine-rich domain; DSL, Delta/Serrate/Lag-2; EGF, epidermal growth factor-like repeats; ICN, intracellular activated form of Notch; LNR, 3 Lin/Notch repeats; NBD, NF-κB binding domain; NLS, nuclear location sequence; PEST, proline-glutamate-serine-threonine-rich domain; RAM, RBP-J association molecule; TAD, transcriptional activation domain; TM, transmembrane domain.

I.4.2 Notch processing and signalling

The Notch precursor is proteolytically cleaved into two fragments, one containing most of the extracellular domain and the other containing the transmembrane region and the intracellular domain. The cleavage is mediated by furin-like convertases in the trans-Golgi compartment (Blaumueller *et al.*, 1997; Logeat *et al.*, 1998). The two fragments are non-covalently linked to form the heterodimeric mature Notch receptor found on the plasma membrane. The glycosyltransferase Fringe modifies the extracellular EGF repeats in the Golgi, which most probably regulates the specificity of Notch for its ligands (Bruckner *et al.*, 2000; Hicks *et al.*, 2000; Ju *et al.*, 2000; Moloney *et al.*, 2000). Three vertebrate homologues of *Drosophila* Fringe have been identified with different expression patterns: Lunatic Fringe, Manic Fringe, and Radical Fringe. Ligand binding at the cell surface is thought to induce endocytosis and a conformational change in the Notch heterodimer, exposing an extracellular site just outside the transmembrane domain to ADAM metalloprotease cleavage and shedding of the extracellular portion (Brou *et al.*, 2000). Once the extracellular domain of Notch has been removed, the remaining carboxy-terminal portion consisting of a short extracellular stalk, the transmembrane domain and the complete intracellular domain, is efficiently proteolysed in a Presenilin-dependent manner releasing the intracellular activated form of Notch (ICN; De Strooper *et al.*, 1999; Song *et al.*, 1999b; Struhl and Greenwald, 1999). This domain interacts with transcriptional effectors of the CSL family, resulting in transcriptional activation of target genes such as Enhancer of split (E(spl)).

An isoform of cell surface Notch1 has recently been identified that does not undergo proteolytic processing or heterodimer assembly (Bush *et al.*, 2001). Its activation upon ligand binding is thought to be CBF1-independent. Further analysis will be needed to determine whether this pathway corresponds to the CSL-independent Notch pathway inferred from genetic studies in *Drosophila* and mammalian cells (see below).

ICN interacts with a number of cytoplasmic and nuclear proteins, permitting signal transduction through at least two pathways, one involving CSL proteins and one independent of CSL.

The **CSL-dependent pathway** involves association of intracellular domain of Notch with the CSL protein CBF1, the mammalian homologue of *Drosophila* Su(H), and transcriptional activation of Hairy/Enhancer of Split (Hes) through binding of Notch/CBF1 to the Hes promoter (Jarriault *et al.*, 1995). The precise molecular mechanisms of how Notch can transactivate CBF1-regulated genes are unknown. CBF1 is ubiquitously expressed in most mammalian cells. In the absence of activated Notch, it functions as a transcriptional repressor and has been shown to interact with the corepressor/histone deacetylase complex (HDAC) (Fig. I.9; Hsieh *et al.*, 1999; Kao *et al.*, 1998; Kurooka and Honjo, 2000; Oswald *et al.*, 1998). ICN is thought to replace HDAC and possibly recruit coactivator/histone acetyltransferases (HAT) or unknown transcription factors resulting in the expression of Notch target genes. *Drosophila* Mastermind and its human homologue Mastermind-like-1 (MAML1) are transcriptional coactivators of Notch signalling, which bind to the ANK repeats of ICN and have been shown to stabilise the ICN-CBF1 complex (Schuldt and Brand, 1999; Wu *et al.*, 2000). The negative regulator Numb inhibits Notch signalling, probably by preventing ICN translocation into the nucleus (Frise *et al.*, 1996). However, it is not known whether Numb is expressed in haematopoietic cells.

CSL-independent signalling also results in transcriptional regulation, but is mediated by different effector molecules, such as Deltex, and may regulate distinct target genes (Matsuno *et al.*, 1997; Nofziger *et al.*, 1999; Ordentlich *et al.*, 1998; Shawber *et al.*, 1996). Three closely related members of the Deltex family (Deltex1-3) have also been identified in mammals (Kishi *et al.*, 2001; Matsuno *et al.*, 1998; Pampeno and Meruelo, 1996). Deltex has three distinct motifs: a binding site for ICN, a SH3 binding domain and a zinc-finger domain, which is known to be involved in protein-protein interactions in various systems (Freemont, 1993; Matsuno *et al.*, 1995). The SH3 binding domain of Deltex is shown to interact with the adapter protein Grb2 in a yeast two-hybrid system (Matsuno *et al.*, 1998). However, it is not known if the SH3 binding domain of Deltex can link ICN and Grb2 *in vivo*. Deltex is a positive regulator of Notch signalling (Diederich *et al.*, 1994; Matsuno *et al.*, 1995). However, the mechanism of Notch regulation by Deltex has remained largely unknown.

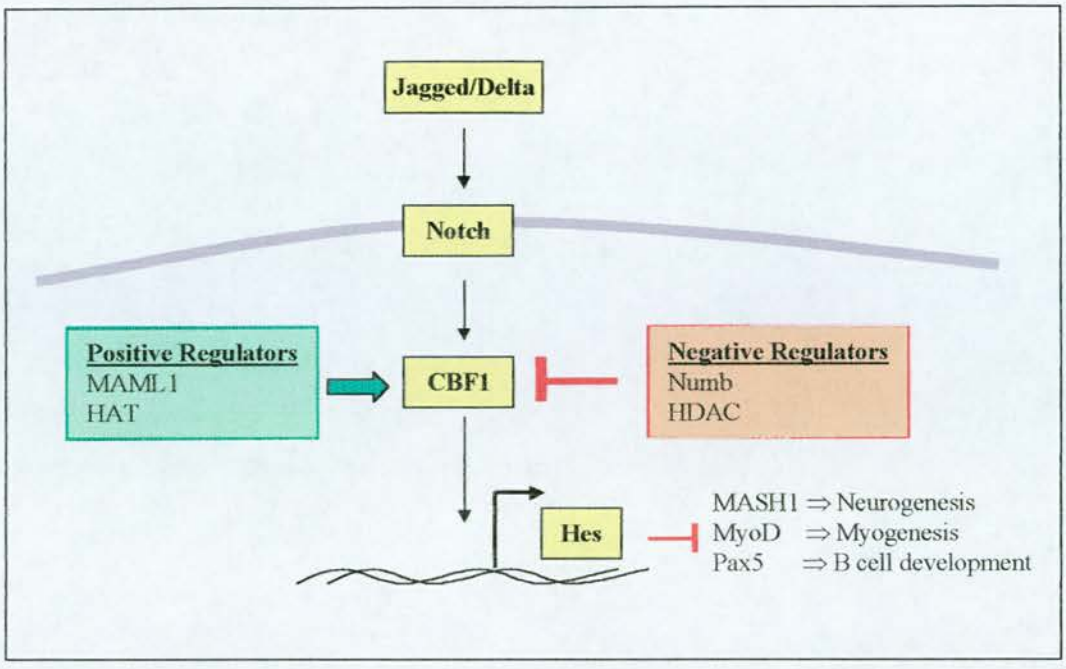


Figure I.9. Regulators of CBF1-dependent Notch signalling.
Ligand-induced Notch activation inhibits cell differentiation in neurogenesis myogenesis and in B cell development by repressing cell-lineage specific genes MASH1, MyoD and Pax5, respectively. CBF1, C promoter binding factor 1; HAT, histone acetyltransferases; HDAC, histone deacetylase complex; Hes, hairy/enhancer of split; MAML1, Mastermind-like-1; MASH1, mammalian achaete-scute homologue-1

I.4.3 Targets of Notch signalling

The ICN-CBF1 complex induces transcription of the Hes genes, the mammalian homologues of E(spl) (Fig. I.9 & I.10). Hes proteins are basic helix-loop-helix (bHLH) transcription factors and inhibit activity of other bHLH proteins, thereby suppressing transcription of lineage-specific genes. There are seven described members in the mammalian Hes family. Among these seven, only Hes1, Hes5, Hes6 and Hes7 are known to be involved in Notch signalling (Table I.3; Bae *et al.*, 2000; Bessho *et al.*, 2001; Ohtsuka *et al.*, 1999).

Hes1 is known to inhibit the DNA-binding activity of E47, a product of the E2A gene (Sasai *et al.*, 1992). MyoD and MASH1, two transcription factors important in myogenesis and neurogenesis, respectively, interact with E47 and activate transcription. Hes1 also inhibits the transcriptional activity of these factors probably by depleting E47 from the complex (Kuroda *et al.*, 1999; Sasai *et al.*, 1992). A bHLH protein in *Drosophila* related to MASH1, the

Achaete-Scute complex (Ac-Sc), is inhibited by Notch, and recent studies in rat neuroblasts have shown that Hes1 also inhibits MASH1 expression (Castella *et al.*, 1999). Hes proteins interact with transducin-like E(spl) (TLE) proteins, the mammalian counterparts of *Drosophila* Groucho, corepressors likely to be important in Hes-mediated transcriptional repression (Grbavec and Stifani, 1996). Recent data suggests that Groucho negatively regulates the transcription factor Pax5, which is required for B cell development (Eberhard *et al.*, 2000). Interaction of Hes1 with other Hes members may be important. Hes6 does not have DNA binding activity but directly binds to Hes1 and inhibits its function (Bae *et al.*, 2000).

Targets of the CSL-independent pathway are less characterised. Deltex interferes with the Ras signalling pathway and inhibits E2A-encoded transcription factors (Fig. I.10; Ordentlich *et al.*, 1998; Pui *et al.*, 1999), which will be described in more detail in section I.4.5.2.

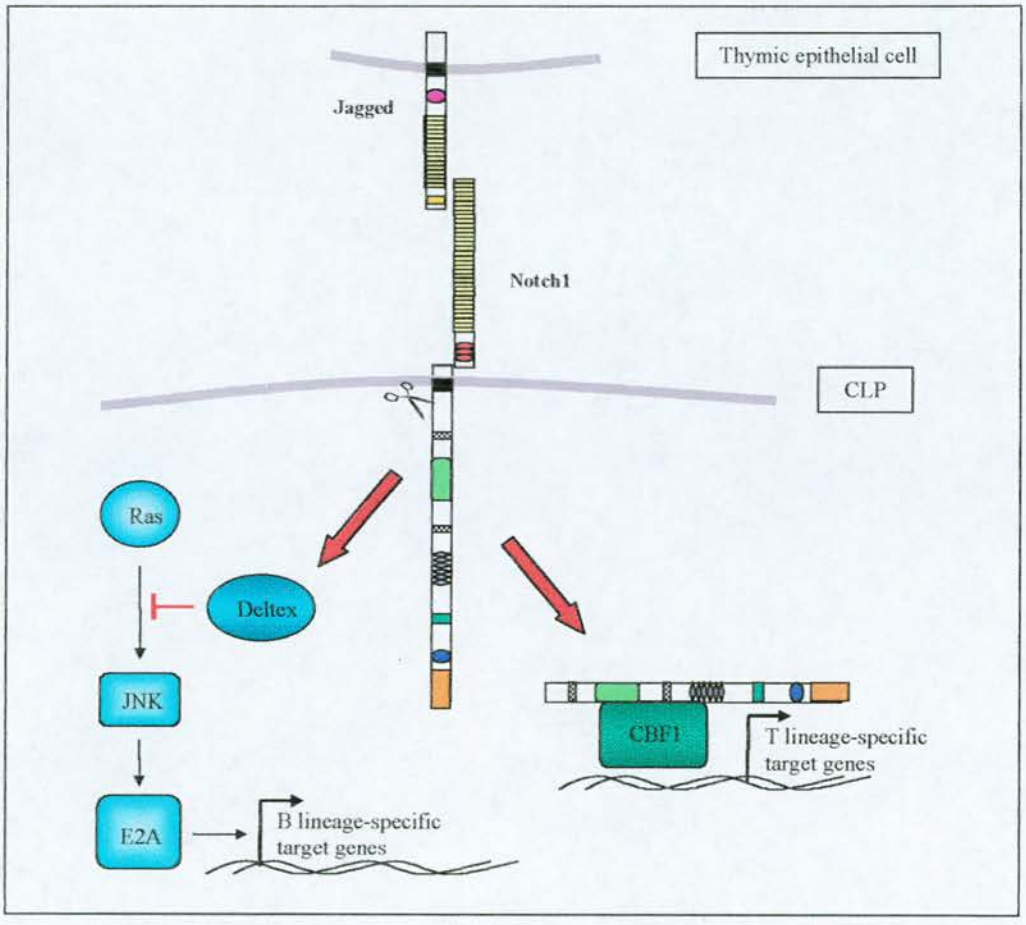


Figure I.10. Molecular control of T cell fate specification by Notch1 signalling.
In this hypothetical model, the interaction of ligands such as Jagged1 and/or Jagged2 expressed on thymic epithelial cells with the Notch1 receptor expressed on common lymphoid precursors (CLPs) leads to activation and cleavage of the Notch1 receptor at (or close to) its transmembrane domain. Two signalling pathways are simultaneously activated. Translocation of the intracellular subunit of

the Notch1 receptor into the nucleus and its heterodimerisation with RBP-Jκ converts RBP-Jκ from a transcriptional repressor into an activator, thereby leading to transcription of T-lineage-specific target genes (still to be identified) and instruction of CLPs towards the T-cell lineage. Simultaneously, a second pathway is activated through Deltex leading to inhibition of E2A-encoded transcription factors such as E47, which are essential for B-cell development. This pathway involves the inhibition of Ras-activated JNK.

I.4.4 Lateral specification/inhibition and lateral induction

Notch receptors mediate two different processes: lateral specification/inhibition and lateral induction. Notch signalling can occur either among a group of equivalent cells (homotypic interactions) or between non-equivalent cells (heterotypic interactions), both of which are essential during development.

I.4.4.1 Homotypic interactions: lateral specification/inhibition

Lateral specification events are often responsible for the segregation of specific lineages from clusters of precursor cells.

In a group of initially equivalent cells, stochastic events and/or extrinsic factors single one cell out to express more Notch ligands than the others (Fig. I.11). This cell signals to neighbouring cells through Notch. Activation of Notch in the receiving cells inhibits expression of lineage-commitment gene products thereby inhibiting their differentiation (lateral inhibition). Additionally, Notch signalling inhibits production of the Notch ligand. Consequently, a cell that produces more ligand forces its neighbours to produce less. This enables the cell to increase its ligand production even further, because it receives a weakened inhibitory signal back from its neighbours. The effect of this feedback loop is to drive neighbouring cells into different developmental pathways: any initial difference between them is intensified and maintained.

The most thoroughly studied examples of Notch signalling are in the control of neurogenesis in *Drosophila* and in vertebrates (Fig. I.11). Notch signalling here controls the commitment of equivalent progenitor cells to differentiate. The major target gene for Notch signalling in *Drosophila* neurogenesis is the Ac-Sc complex, which is essential for the differentiation of progenitors into neural cells. Notch suppresses the Ac-Sc complex in neural progenitors

through E(spl) and thereby inhibits their differentiation into neurons (Heitzler *et al.*, 1996). Because Ac-Sc is also a transcriptional activator of Delta (Kunisch *et al.*, 1994), Delta expression is suppressed in response to Notch activation. Therefore, the nascent neurons, by expressing Delta, deliver lateral inhibition to the Notch1-expressing progenitors to prevent them from differentiating prematurely into neurons. This lateral inhibition mechanism has been confirmed in *Xenopus* (Chitnis *et al.*, 1995), chick (Austin *et al.*, 1995; Henrique *et al.*, 1997), mouse (de la Pompa *et al.*, 1997) and zebrafish (Dornseifer *et al.*, 1997). Many cells during development can choose a "default" pathway of differentiation (neurons in the case of neurogenesis), and in general, Notch is thought to inhibit selection of the default cell fate, rather than promote selection of an alternate fate.

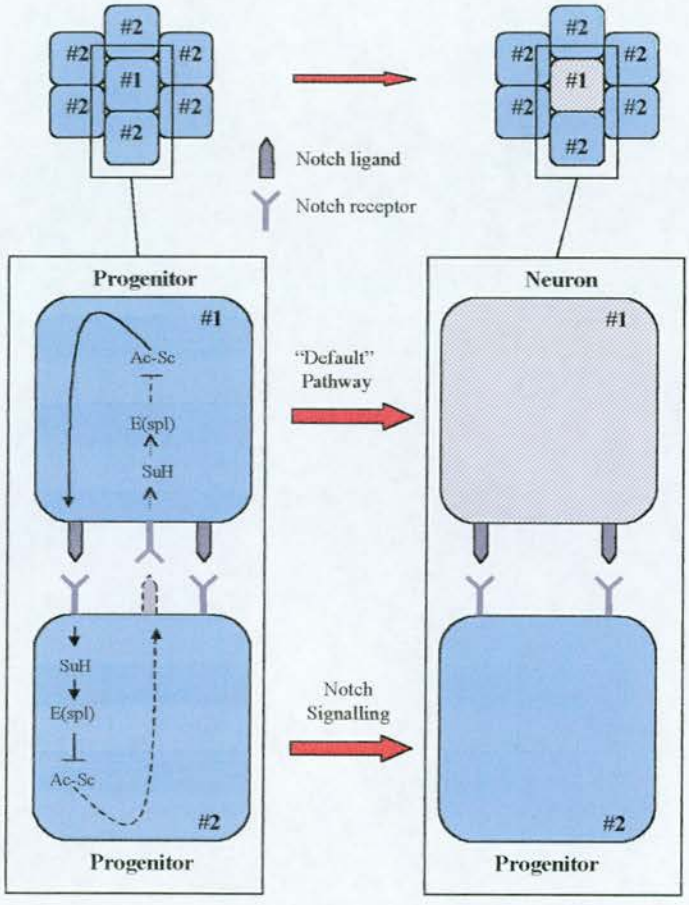


Figure I.11. Neurogenesis in *Drosophila* as an example of lateral inhibition.
All cells in a cluster of equipotent cells initially express the same amount of Notch, Su(H), E(spl), Ac-Sc and Delta. A small stochastic variation in Delta expression of one cell (#1) stimulates Notch in the neighbouring cells (#2) leading to E(spl) activation that represses Ac-Sc activity and thereby lineage-commitment and Delta expression. Cell #1 does not receive any inhibitory signals through Notch from neighbouring cells (#2) anymore and differentiate along the primary or "default" pathway into a neuron.

I.4.4.2 Heterotypic interaction: lateral induction

Notch signalling also occurs between Notch and Notch ligands expressed on different cell types (Fig. I.12). Inductive signalling through these heterotypic interactions is regulated primarily by ligand expression, limiting Notch activation to those cells in direct contact with ligand-expressing cells. Interactions between Notch receptors and ligands can also be modulated by other molecules, such as Fringe (Fleming *et al.*, 1997; Panin *et al.*, 1997), and further regulated by a feedback loop, which results in increased ligand expression (de Celis and Bray, 1997). Inductive signalling thus permits the establishment of finely demarcated boundaries between cell types, exemplified by dorsal-ventral boundary formation during fly wing margin specification and vertebrate limb development (Ju *et al.*, 2000).

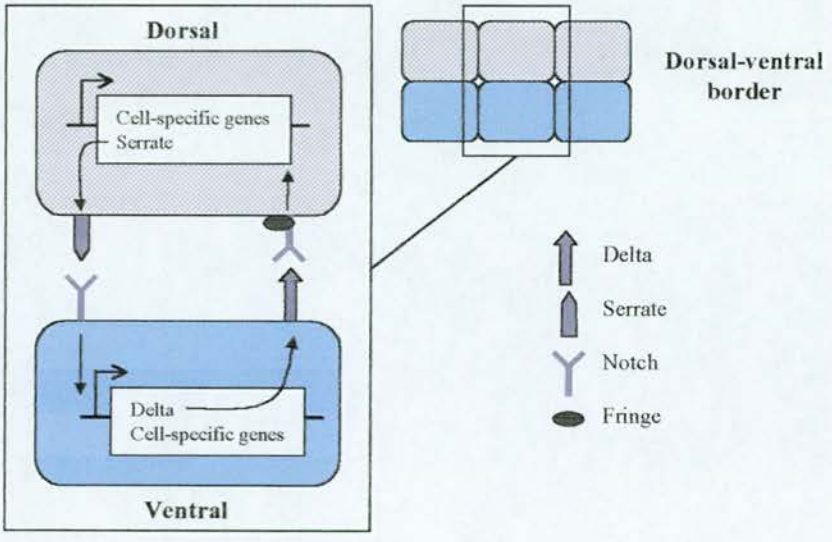


Figure I.12. Development of the dorsal-ventral border of *Drosophila* wing as an example of lateral induction.
Serrate signals from dorsal cells to Notch on ventral cells and is inhibited from signalling to Notch on other dorsal cells by Fringe. Delta signalling from ventral cells to dorsal cells is potentiated by Fringe associated with dorsal Notch. The expression of the ligands is maintained by a positive feedback loop as Serrate signalling to Notch in ventral cells induces expression of Delta, while Delta signalling to Notch in dorsal cells induces expression of Serrate.

I.4.5 Notch signalling in the immune system

I.4.5.1 Notch signalling and myelopoiesis

Expression of Notch receptors and ligands was observed in cells and tissues of the immune system and there is accumulating evidence that endogenous Notch signalling regulates the differentiation of immune cells. Notch is expressed in human and mouse $CD34^+$ haematopoietic precursors (Milner *et al.*, 1994; Varnum-Finney *et al.*, 1998). Notch ligands are expressed on bone marrow stromal cells as well as in haematopoietic precursors (Han *et al.*, 2000; Jones *et al.*, 1998; Karanu *et al.*, 2001; Li *et al.*, 1998; Nomaguchi *et al.*, 2001; Tsai *et al.*, 2000). Many groups have made use of myeloid progenitor cell lines as a model system for haematopoietic differentiation (Migliaccio *et al.*, 1989): 32D cells proliferate as blasts in the presence of IL-3, but they differentiate and undergo granulopoiesis in the presence of G-CSF or GM-CSF. Coculture with Notch ligands or transfection with activated Notch inhibits differentiation of 32D cells and $CD34^+$ haematopoietic progenitors (Carlesso *et al.*, 1999; Jones *et al.*, 1998; Li *et al.*, 1998; Milner *et al.*, 1996; Varnum-Finney *et al.*, 1998) rather than promotes their differentiation (Schroeder and Just, 2000a; Schroeder and Just, 2000b; Walker *et al.*, 1999). Additionally, Notch activation promotes survival and expansion of haematopoietic precursors *in vitro* and *in vivo* (Han *et al.*, 2000; Karanu *et al.*, 2001; Tsai *et al.*, 2000), consistent with the ability of ICN1 to induce immortalised, cytokine-dependent progenitor cell lines from murine bone marrow cells (Varnum-Finney *et al.*, 2000). In other situations, however, ICN1 has been inhibitory for proliferation (Schroeder and Just, 2000a; Schroeder and Just, 2000b). The inhibition of differentiation by Notch is consistent with the lateral inhibition model (section I.4.4.1), whereby Notch activation retains the cells in an undifferentiated pluripotent state. However, in the haematopoietic system, Notch interactions may be either homotypic or heterotypic, since both haematopoietic precursors and stromal cells express Notch ligands. Different extrinsic factors such as cytokines or growth factors may be responsible for distinct regulatory mechanisms and increase the diversity of Notch function.

It has been reported that Notch1 inhibits myeloid differentiation of 32D cells in response to G-CSF, whereas Notch2 inhibits in response to GM-CSF (Bigas *et al.*, 1998). This cytokine specificity is associated with the NCR region mentioned above (Fig. I.8). Furthermore, Delta1 promotes differentiation of bone marrow cells to myeloid DCs at the expense of other lineages

in the presence but not absence of GM-CSF (Mizutani *et al.*, 2000; Ohishi *et al.*, 2001) and induces apoptosis in peripheral blood monocytes cultured with M-CSF, but not GM-CSF (Ohishi *et al.*, 2000). Notch signalling promotes enhanced entry of 32D cells in the presence of G-CSF into granulopoiesis and increased survival in a CBF1-dependent manner. Elevated numbers of viable cells during G-CSF treatment were also observed in 32D cells overexpressing Hes1, consistent with activation of the CBF1 pathway (Tan-Pertel *et al.*, 2000).

I.4.5.2 Notch signalling and lymphopoiesis

BM-derived common lymphoid progenitors (CLPs) give rise to either B or T cells, in addition to NKs and lymphoid dendritic cells (see section I.1.1). Following commitment to the T-cell lineage, immature pro-T cells begin to rearrange and express their TCRγ, δ and β genes (Kisielow and von Boehmer, 1995). Pro-T cells that successfully rearrange TCRγ and TCRδ will express a TCRγδ and be eligible to develop further as γδT cells. By contrast, productive TCRβ gene rearrangement will lead to expression of a pre-TCR (consisting of a TCRβ chain associated with an invariant pTα chain), which is compatible with further development as αβT cells. αβ lineage-committed pre-T cells expand and progress to the $CD4^+CD8^+$ double-positive (DP) stage of development. At this point, successful TCRα rearrangement leads to expression of a TCRαβ that can be positively or negatively selected as DP thymocytes progress to the mature $CD4^+CD8^-$ single-positive ($CD4^+$ SP) and $CD4^-CD8^+$ SP ($CD8^+$ SP) stages. The TCRαβ on mature $CD8^+$ SP and $CD4^+$ SP cells recognises peptides presented by MHC class I or class II, respectively. Therefore, it is possible that specific TCRαβ signalling (in conjunction with related signalling through Lck-associated CD4 or CD8 coreceptors) is sufficient in itself to direct DP cells to the $CD4^+$ or $CD8^+$ T cell lineage. Alternatively, it is possible that CD4/CD8-lineage determination in DP cells occurs independently of TCR-MHC interactions, and that the latter are required only to ensure the survival of cells with an appropriate match of coreceptor and TCR specificity.

T/B cell-lineage commitment

Recent studies indicate that Notch signalling controls the lineage choice between B and T cell fates. Mice with conditional inactivation of the Notch1 gene in BM precursors display a block in T cell development at an early stage, before expression of T cell lineage markers. $B220^+$

cells, however, develop in the thymus of these mice (Radtke *et al.*, 1999; Wilson *et al.*, 2000). This suggests that early lymphoid precursors may choose a B cell fate in the absence of Notch1 signalling. Similar results were obtained with chimeras in which Hes1 null foetal liver was injected into irradiated RAG2 null mice: B cells developed normally while thymic T cell development was arrested at the $CD4^-CD8^-$ double negative (DN) stage (Tomita *et al.*, 1999). Further, it has been shown that expression of constitutively active Notch1 in bone marrow precursors using retroviral mediated gene transfer results in the development of immature DP T cells in the bone marrow. This completely prevents development of B220 cells (Pui *et al.*, 1999).

Taken together, these complementary gain-of-function and loss-of-function studies clearly indicate that Notch1 provides a critical signal that determines the outcome of a binary (T or B) cell fate decision by a common lymphoid progenitor (CLP, Fig. I.10 & I.13). By analogy with the role of Notch genes in invertebrate systems, one interpretation of these data would be that B cell development from the CLP might represent the "default" pathway that occurs in the absence of Notch1 signalling (Fig. I.11). Alternatively, BM cells, which express several ligands (Jones *et al.*, 1998; Li *et al.*, 1998), may induce CLPs to choose the T cell fate (Fig. I.12).

The mechanism by which Notch1 controls T/B-cell-fate specification in a CLP remains unclear. Notch activation in T cell precursors induces transcription of Hes1 (Chang *et al.*, 2000), which is consistent with the finding that CBF1 is associated with ICN in T cells but not in B cells (Callahan *et al.*, 2000). However, the CBF1-independent pathway involving Deltex also plays an important role in suppressing B cell development. Signals through the Notch/Deltex pathway can result in repression of E47 (Fig. I.10), which is a bHLH transcription factor encoded by the E2A gene (Ordentlich *et al.*, 1998; Pui *et al.*, 1999). E47 is required for proper B cell development (Bain *et al.*, 1997) and is sufficient to activate transcription of the Ig heavy-chain gene locus (Schlissel *et al.*, 1991). In addition to the inhibition of E2A-dependent transcription (Pui *et al.*, 1999), constitutively activated Notch1 and Notch2 repress Ig heavy-chain gene transcription (Morimura *et al.*, 2001). The mechanism leading to repression of E47 activity is thought to occur via inhibition of Ras and/or JNK signalling (Ordentlich *et al.*, 1998). In *Drosophila*, Su(H)-independent Notch signalling has already been shown to downregulate signalling through the JNK pathway (Zecchini *et al.*, 1999). Thus, in addition to an instructive role in promoting T-cell-lineage commitment via CBF1, Notch1 might simultaneously repress the expression of B-lineage-specific genes via Deltex.

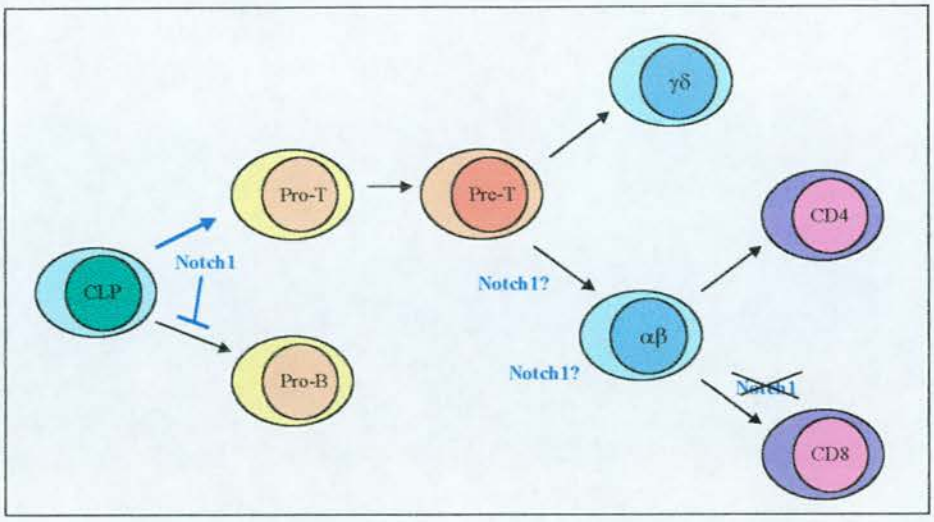


Figure I.13. Notch1 signalling during T cell development.
Common lymphoid progenitors (CLPs) make a first cell-fate decision giving rise to either pro-B cells or pro-T cells. The DN pro-T cell then matures further to a pre-T-cell stage, where commitment to either αβ or γδT cell lineage occurs. Finally, αβ lineage committed DP thymocytes must choose between the mature $CD4^+$ or $CD8^+$ T cell fates. Gain-of-function studies suggest a decisive role for Notch1 in T/B- and αβ-lineage decisions, whereas recent loss-of-function studies support a more restricted role for Notch1 in T cell development. The role of Notch signalling in survival and differentiation of DP thymocytes is much debated (see discussion below).

αβ/γδ T cell-lineage commitment

Notch activity has also been suggested to control the αβ versus γδ lineage choice (Washburn *et al.*, 1997) as well as the $CD4^+$ versus $CD8^+$ lineage choice during thymocyte development (Robey *et al.*, 1996). The experiments looking at the effect of Notch activity on αβ versus γδ lineage choice used mice with a chimaeric haematopoietic system populated by a mixture of $Notch1^{+/-}$ and $Notch1^{+/+}$ cells. $Notch1^{+/-}$ BM precursors contribute less to the αβ lineage than $Notch^{+/+}$ precursors. Also, overexpression of ICN1 under the control of the proximal Lck promoter in transgenic mice overcomes the block in αβT cell development imposed by the absence of TCRβ. Collectively, these data suggest that Notch1 signalling favours the development of αβT cells at the expense of γδT cells (Washburn *et al.*, 1997). However, this was debated by Kaneta *el al.* because Hes1-deficient thymocytes did not display a perturbed αβ/γδ T cell ratio (Fig. I.13; Kaneta *et al.*, 2000). These results suggest a possibility that Notch1 signal and Hes1 signal are not always identical, at least in developing T cells.

CD4$^+$/CD8$^+$ T cell-lineage commitment

The first evidence for a role of Notch in CD4$^+$/CD8$^+$ cell fate decision came from experiments conducted with transgenic mice carrying the activated Notch1 transgene under the control of the Lck promoter (Robey *et al.*, 1996). These mice have increased numbers of CD8$^+$ thymocytes and a decreased number of CD4$^+$ T thymocytes. However, these T cells were short-lived and could not be detected in secondary lymphoid tissue. Consistent with Robey's data, Kim and Siu found that overexpression of Hes1 or ICN1 silences CD4 transcription (Kim and Siu, 1998).

The interpretation of these results has been challenged by several groups. Deftos and colleagues found that transgenic mice expressing ICN1 transgene under the control of the Lck promoter promoted the maturation of DP thymocytes into both CD4$^+$ and the CD8$^+$ SP thymocytes (Deftos *et al.*, 2000). The reason for the differences between these two lines of transgenic mice, both expressing ICN1 under the control of the Lck promoter, is not clear. However, Robey *et al.* were using a slightly shorter form of ICN1, which lacks the C-terminal portion TAD, which is important for enhancing Notch function (Fig. I.8; Aster *et al.*, 2000).

Lethally irradiated mice reconstituted with BM cells retrovirally transduced with the same ICN1 transgene used by Deftos and colleagues did not develop SP thymocytes (Izon *et al.*, 2001). Instead a developmental arrest at the DP stage was observed. Izon *et al.* argued that the Lck promoter used in the previous reports decreases in the activity during DP thymocytes maturation (Allen *et al.*, 1992), whereas the retroviral LTR promoter is active throughout T cell development. Therefore, ICN1 in the previous reports was likely to be expressed at a lower functional dose explaining the different outcome. Pear's group extended these results further by showing that Notch1 is required for T cell commitment but insufficient to rescue a block in pre-TCR signalling necessary for either T cell expansion or DP development (Allman *et al.*, 2001; Pui *et al.*, 1999).

In summary, several reports have implicated Notch1 signalling either in the CD4/CD8-lineage commitment event or in the subsequent maturation and survival of CD4$^+$ SP and/or CD8$^+$ SP cells. These studies (summarised in Table I.4) are largely based on gain-of-function approaches in which Notch IC is overexpressed.

However, a recent report debated the involvement of Notch1 in the development of DP or SP thymocytes. Inactivation of Notch1 in DN thymocytes did not perturb the development of mature CD4$^+$ and CD8$^+$ T cells (Wolfer *et al.*, 2001). Additionally, in contrast to conclusions reached using thymoma cell lines (Table I.4; Deftos *et al.*, 1998; Jehn *et al.*, 1999), Notch1

deficiency in DP thymocytes does not affect their sensitivity to spontaneous or glucocorticoid-induced apoptosis.

The discrepancy between Wolfer's data and earlier reports may be explained by the activation of signalling pathways by ICN1 that are not normally controlled by Notch1 under physiological conditions. For example, Notch IC might activate signalling that is normally mediated via Notch2 or Notch3, both of which are expressed in the thymus (Felli *et al.*, 1999). In this context, overexpression of the cytoplasmic domain of Notch3 in immature thymocytes of transgenic mice under the control of the Lck promoter does not affect $CD4^+/CD8^+$ lineage commitment, although it does result in increased expansion of thymocytes leading to T cell lymphomas (Bellavia *et al.*, 2000).

Table 1.4. Evidence for Notch1 influences on $CD4^+/CD8^+$ lineage commitment, maturation and survival.

Experimental system	Result	Reference
Transgenic mice overexpressing ICN1 under the control of the Lck promoter	Selective increase in $CD8^+$ SP thymocytes	(Robey *et al.*, 1996)
Transgenic mice overexpressing ICN1 under the control of the Lck promoter	Increased maturation of $CD4^+$ and $CD8^+$ SP thymocytes	(Deftos *et al.*, 2000)
Mice reconstituted with BM cells overexpressing ICN1	Developmental arrest of thymocytes at the DP stage	(Izon *et al.*, 2001)
Mice reconstituted with BM cells overexpressing ICN1	Immature DP T cells in the BM	(Pui *et al.*, 1999)
Mice reconstituted with BM cells from $RAG^{-/-}$ mice overexpressing ICN1	Immature DN T cells in the BM	(Allman *et al.*, 2001)
Thymoma transfected with ICN1	Increased resistance to glucorticoid-induced apoptosis	(Deftos *et al.*, 1998)
Thymoma transfected with ICN1	Increased resistance to activation-induced apoptosis	(Jehn *et al.*, 1999)
Notch1 inactivation in BM cells *in vivo*	Accumulation of B cells in the thymus	(Radtke *et al.*, 1999)
Notch1 inactivation in DN thymocytes *in vivo*	No effect on subsequent T cell development	(Wolfer *et al.*, 2001)
Antisense Notch1 in FTOC	Decreased maturation of $CD8^+$ SP thymocytes	(Yasutomo *et al.*, 2000)

FTOC: Foetal thymus organ culture

I.4.6 Concluding remarks

In conclusion, Notch signalling has been shown to interfere with the Ras/JNK signalling pathways (Ordentlich *et al.*, 1998; Zecchini *et al.*, 1999) and NF-κB signalling (see section III.1). Additionally, Notch1 activation inhibits TCR-mediated or PMA/ionomycin-induced NF-AT/AP1 promoter activity in Jurkat cells (Izon *et al.*, 2001). These pathways are crucial during T cell activation. Therefore, it seems likely that Notch signalling is able to modulate a peripheral T cell response, provided that $CD4^+$ and $CD8^+$ T cells do express components of the Notch pathway.

Indeed, a role for Notch signalling in the differentiation of $CD4^+$ T cells into regulatory T cells has been reported by our laboratory. Murine APCs retrovirally transduced with human Jagged1 and pulsed with peptide were transferred into naïve mice, which were subsequently challenged to induce productive immunity. T cells from these mice were hyporesponsive to the antigen *in vitro* and, upon adoptive transfer into naïve mice, induced antigen-specific tolerance *in vivo* (Hoyne *et al.*, 2000). Furthermore, regulatory $CD4^+CD25^+$ T cells differentially regulate Delta1, Notch4, Hes1 and Deltex upon TCR stimulation, whereas their CD25-negative counterparts do not (Ng *et al.*, 2001).

I.5 Aims

Notch plays a crucial role in both the development and differentiation of haematopoietic cells. It acts by activating or inhibiting lineage-specific genes and affects several signalling pathways that are crucial during the induction of an immune response such as the NF-κB or the MAPK pathway. It has been shown that APCs transfected with Jagged1 induce the differentiation of naïve T cells into regulatory T cells *in vivo*, suggesting an important role for the Notch pathway in immunity (Hoyne *et al.*, 2000). Therefore, the aim of my PhD was to investigate the role of Notch signalling during the induction of an immune response.

The question that I addressed was whether and to what extent the components of the Notch signalling pathway are expressed in peripheral $CD4^+$ and $CD8^+$ T cells of naïve mice (chapter III). It has been reported that Notch can activate or inhibit NF-κB activity (Bellavia *et al.*, 2000; Guan *et al.*, 1996; Wang *et al.*, 2001) and that activation of NF-κB can induce the expression of the Notch1 and Jagged1 (Bash *et al.*, 1999; Li *et al.*, 2001). To investigate whether Notch components are differentially expressed during NF-κB activation in T cells, expression of Notch receptor, ligands and downstream components was examined in $CD4^+$ and $CD8^+$ T cells activated by ligation of their TCR and CD28 or by PMA/ionomycin stimulation. Further, Notch activation in T cells during a mixed lymphocyte reaction (MLR) was studied, by using a MHC class II-transfected murine fibroblast cell line co-transfected with the Notch ligands Jagged1 or Delta1 (chapter V). The influence of Notch signalling on proliferation, cytokine production, phenotype and apoptosis in activated T cells was also investigated.

APCs play an instructive role during an immune response. They have the capacity to activate, anergise, polarise and even to kill T cells. Many soluble and membrane-bound molecules have been identified in APCs to be important for these events. However, a lot remains unclear such as, for example, the molecular mechanism by which immature or IL-10 treated DCs induce regulatory or anergic T cells, respectively. Expression of Notch components in DCs was therefore investigated (chapter IV). T cells are able to condition or "license" DCs (Ridge *et al.*, 1998; Vendetti *et al.*, 2000). Thus T cells are able to instruct DCs and vice versa. If Notch is involved at the DC-T interphase, it is important to determine whether signalling is bi- or unidirectional and in the latter case, to identify the signalling and the receiving cell by looking at the induction of downstream target, such as the Hes genes, of Notch signalling.

Finally, transgenic mice have been generated with inducible expression of Jagged1 or Delta1 in DCs (chapter VI). These mice will allow the future study of Notch signalling during an immune response *in vivo*.

II Materials & Methods

II.1 Materials and Buffers

- CFSE (5-(and –6)-carboxyfluorescein diacetate succinimidyl ester)

CFSE (Molecular Probes) was dissolved to a concentration of 5mM in DMSO. Aliquots were prepared and stored at -20°C.

- Complete HAT+MX Culture Media ± Geneticin

500mls RPMI 1640 with 10% heat-inactivated foetal calf serum (FCS), 100IU/ml penicillin/streptomycin (Gibco), 2mM L-glutamine (Gibco), 15μg/ml hypoxanthine (Gibco), 0.2μg/ml aminopterin (Gibco), 5μg/ml thymidine (Gibco), 6μg/ml mycophenolic acid and 250μg/ml xanthine ± 500μg/ml geneticin (Gibco). Kept at 4°C.

- Complete Iscoves culture media

500mls Iscoves 1640 (Gibco) with 10% foetal calf serum (FCS) [heat inactivated], 50μM β–mercaptoethanol (ME), 100U/ml penicillin/streptomycin (Gibco), 2mM L-glutamine (Gibco). Kept at 4°C.

- Complete RPMI culture media

500mls RPMI 1640 with 10% foetal calf serum (FCS) [heat inactivated], 50μM β-mercaptoethanol (ME), 100U/ml penicillin/streptomycin (Gibco), 2mM L-glutamine (Gibco). Kept at 4°C.

- DC-RPMI

As complete RPMI but without the addition of β–mercaptoethanol (ME). Kept at 4°C.

- EDTA/Trypsin

1x Trypsin-EDTA (0.5μg/ml trypsin 0.2μg/ml EDTA, Gibco). Aliquots were prepared and stored at -20°C.

- ELISA Blocking Buffer

PBS containing 1% BSA, 5% sucrose and 0.05% Tween-20. Kept at 4°C.

- ELISA Reagent Diluent

For IL-2 and IFNγ ELISAs: 20mM Trizma base 150mM NaCl containing 0.05% Tween-20 and 0.1% BSA. For IL-4 and IL-10 ELISAs: PBS containing 1% BSA. Kept at 4°C.

- ELISA Stop Solution

0.5M H_2SO_4. Kept at RT.

- ELISA Substrate Solution

TMB Liquid Substrate System for ELISA. Kept at 4°C.

- ELISA Wash Buffer

PBS containing 0.05% Tween-20. Kept at room temperature (RT).

- FACS Buffer

10% heat-inactivated foetal calf serum (FCS) in PBS, 0.1% NaN_3. Kept at 4°C.

- FACS Fixing Buffer

2% formaldehyde in PBS. Kept at 4°C.

- Fixing Buffer for intracellular cytokine staining

4% paraformaldehyde in PBS. Kept at 4°C in the dark.

- Gey's Red Blood Cell Lysis Buffer

155mM $KHCO_3$ and 10mM NH_4Cl, 0.1mM EDTA (Gibco). Kept at 4°C.

- Luria-Bertani (LB) broth (50ml)

One LB Broth Tablet (Sigma) was diluted in 50ml distilled water. Sterilised by autoclaving. Stored at RT.

- Luria-Bertani (LB) agar (50 ml)

One LB Agar Tablet (Sigma) was added to 50ml of distilled water and heated to dissolve prior to autoclaving for sterilisation. Stored at RT.

- MACS Buffer

0.5% BSA in PBS. Kept at 4°C.

- PBS/Tween Buffer

PBS with 0.1% Tween-20. Kept at room temperature.

- 6x PCR Loading Buffer (Sambrook *et al.*, 1989)

0.25% weight/volume of bromophenol blue and 40% weight/volume of sucrose in distilled water. Stored at 4°C.

- Permeabilisation Buffer

2% FCS in PBS containing 0.1% saponin, 0.1% NaN_3 and 5mM EDTA (Gibco). Kept at 4°C.

- Preparation of mitogens

Escherichia coli (*E.coli*) lipopolysaccharide (LPS, serotype 055:B5, SIGMA) powder was reconstituted to 1mg/ml in complete RPMI medium. Phorbol 12-myristate 13-acetate (PMA) was reconstituted to 1mg/ml in DMSO. Ionomycin was reconstituted to 5mg/ml in DMSO. Aliquots were prepared and stored at -20°C.

- Recombinant Proteins

Recombinant murine GM-CSF (R&D Systems) was reconstituted in filter-sterilised complete RPMI to a final concentration of 5μg/ml. Recombinant murine TNFα (R&D Systems) was reconstituted in filter-sterilised complete RPMI to a final concentration of 1μg/ml. Recombinant murine IL-10 (R&D Systems) was reconstituted in filter-sterilised complete RPMI to a final concentration of 5μg/ml. Aliquots were prepared and stored at -70°C for up to 3 months.

- TAE Buffer

100ml of 10x TAE Buffer (Gibco) topped up with deionised water to 1 litre.

- TBE (Tris base, boric acid and EDTA) Buffer

100ml of 10x TBE Buffer (Gibco) topped up with deionised water to 1 litre.

II.2 Molecular Biology Techniques

II.2.1 Restriction digests of DNA

0.1-1.0µg DNA, 1µl 10x restriction buffer (Promega Corporation), 1µl restriction enzyme (Promega Corporation) and nuclease-free water (Promega Corporation) were added to give a final volume of 10µl. In case of a double digest, the total volume and the restriction buffer were doubled. If the two restriction enzymes had incompatible buffers, DNA was ethanol precipitated after the first digest and resuspended in sterile distilled water before proceeding with the second digest. After a 2 hrs or overnight incubation at 37°C, 2µl of a 6x loading buffer (40% sucrose, 0.25% bromophenol blue; Sambrook *et al.*, 1989) was added per 10µl total reaction volume. Samples and a 1kb ladder molecular weight marker (Promega Corporation) were run in a 0.8% agarose gel (SeaKem LE agarose, FMC) containing 10µg/ml ethidium bromide in 1x TAE buffer for 1-2 hrs at 80 Volts. DNA fragments were visualised using the UVGrab programme connected to a white/ultraviolet transilluminator (UVP) and isolated by QIAquick gel extraction as described in section II.2.5.

II.2.2 Blunting of cohesive DNA ends

1μl Klenow DNA polymerase (Promega Corporation), 1μl 10x Klenow reaction buffer (supplied), 0.75μl of 20mM dNTPs (supplied) and 7.25μl nuclease-free water (Promega Corporation) were added to 10-20μl of the restriction digest and incubated at 37°C for 30 min.

II.2.3 Dephosphorylation of 5' phosphate of the DNA ends

To prevent compatible or blunt ends from self-religation, the vector was dephosporylated at the 5' end. After restriction digest of the vector, 1μl of 1U/μl shrimp alkaline phosphatase (Roche Boehringer Mannheim) was added per 10μl of the digestion mix. The mixture was incubated at 37°C for 30 min and then at 65°C for 15 min to inactivate the phosphatase.

II.2.4 Preparation of agarose gels

0.8%, 1% or 2% agarose gels (for plasmid DNA, RNA or PCR products, respectively) (SeaKem LE agarose, FMC) containing 0.5μg/ml ethidium bromide (SIGMA) were prepared with 1x TAE buffer (Gibco). The gel was transferred to a gel tank containing 1x TAE buffer. 10μl of the sample was added to 2μl 6x PCR loading buffer and loaded into each well. The agarose gel was run at 80 volts for 1-2 hour. The UVGrab programme connected to a white/ultraviolet transilluminator (UVP) was used to detect the expression of PCR products. PCR marker (50-2,000 bp, SIGMA) or 1kb DNA ladder (0.25-10 kb, Promega Corporation) was used as the molecular weight ladder.

II.2.5 Isolation of DNA from agarose gels

Isolation of DNA products from agarose gels was performed using the QIAquick Gel Extraction Kit (QIAGEN). Briefly, DNA bands of interest were cut from the agarose gel with a sterile blade. 3 volumes of Buffer QC were added and gel slice was dissolved during a 10-min incubation at 50°C. After adding one volume of isopropanol the mixture was applied onto a QIAquick column and centrifuged for 1 min. The DNA bound to the column was washed with 750µl of Buffer PE. After discarding the flow-through, residual buffer was removed by an additional centrifugation for 1min at 10,000 x g. DNA was eluted with 50µl Buffer EB.

II.2.6 Ligation of plasmid vector and insert DNA

After the vector and insert DNA have been prepared for ligation, the insert : vector molar ratio was set approximately at 3-6 : 1 in a small volume to allow insert and vector to anneal together efficiently. 1µl T4 DNA ligase (Promega Corporation) and 1µl 10x ligase buffer (supplied) were added to the insert/vector mixture and nuclease-free water (Promega Corporation) was added to give a final volume of 10µl. The mixture was incubated at 16°C for 3 hrs or overnight. The following controls were included:

	Buffer	Ligase	Plasmid	Insert
Control for restriction efficiency	–	–	+	–
Negative control for religation of the vector	+	+	+	–

II.2.7 Preparation of competent cells and transformation

II.2.7.1 Preparation of competent cells

1ml of overnight *Escherichia coli* (*E.coli*) culture (XLi Blue MRF', Stratagene) was innoculated into 100ml LB broth in a 1-litre flask and shaken for 3 hrs at 37°C. When the

absorbance at 600nm (A_{600}) reached 0.3, 100ml of the cell suspension was aseptically transferred to four 50ml Falcon tubes and chilled for 10 min on ice. After centrifugation (3,000 x g, 10 min, 4°C) supernatant was removed completely, the pellet resuspended in 20ml ice cold 0.1M $CaCl_2$ and kept on ice for 30 min. After centrifugation (3,000 x g, 10 min, 4°C) supernatant was removed completely and the pellet resuspended in 4ml ice cold 0.1M $CaCl_2$.

II.2.7.2 Transformation

10µl DNA was transferred into a sterile 1.5ml Eppendorf tube and 200µl of competent cells are added. After gentle mixing, cells were left on ice for 30 min, then incubated at 42°C for 5 min in a water bath and again left on ice for 2-5 min. 800µl of LB broth freshly supplemented with 20% glucose were added and incubated at 37°C shaking for at least 1.5 hrs. Cells were spun down (10,000 x g, 15 secs, RT), 850µl supernatant was removed and the pellet resuspended in the remaining solution. Transformed cells were spread on the LB plates (section II.2.7.2) and incubated overnight at 37°C. As a positive control, cells were transformed with the original vectors (pBS-CD11c or pBI-EGFP) (control for the efficiency of the transformation). As a negative control, untransformed cells were grown on a selection plate (control for the selection of transformed cells). Transformed colonies were picked, cultured in 5ml LB broth containing 100µg/ml ampicillin at 37°C shaking overnight and plasmid extracted as described in II.2.8. Successful insertion and correct orientation was tested by restriction digest (II.2.1).

II.2.7.3 Preparation of LB plates

LB Agar was heated to melt and then cooled to about 50°C prior to adding ampicillin at a final concentration of 100µg/ml. Agar was poured into bacteriological Petri dishes (Greiner) and stored upside down at 4°C after solidification.

II.2.8 Miniprep purification of plasmid DNA

Purification of plasmid DNA from bacterial cells was done using Promega Wizard Plus SV Minipreps DNA Purification System, according to the manufacturer's instructions. Briefly, 5ml of bacterial culture were harvested by centrifugation at 3,000 x g for 15 min at 4°C. Cells were resuspended in 250µl Cell Resuspension Solution prior to lysis by addition of 250µl Cell Lysis Solution. Cell suspension was incubated for 5 min at RT before adding 10µl of Alkaline Protease Solution. After 5 min incubation at RT, 350µl of Neutralisation Solution was added. The lysate was centrifuged for 10 min at 14,000 x g at RT and cleared supernatant containing solubilised plasmid DNA was transferred into a Miniprep Spin Column by decanting. After 1 min of centrifugation at 14,000 x g at RT, the spin column was washed twice with the supplied Column Wash Solution. Finally, the DNA was eluted in 100µl of RNAse/DNAse-free water (Promega Corporation) and quantified (section II.2.14).

II.2.9 Maxiprep purification of plasmid DNA

Purification of large amounts of plasmid DNA from bacterial cells was done using Quantum Prep Plasmid Maxiprep Kit (Bio-Rad Laboratories), according to the manufacturer's instructions. Briefly, 5ml of bacterial culture were harvested by centrifugation at 3,000 x g for 15 min at 4°C. Cells were resuspended in 15ml of Cell Resuspension Solution prior to lysis by addition of 23ml of Cell Lysis Solution. After 5 min incubation at RT, 15ml of Neutralisation Solution was added. The lysate was then transferred into a QIAfilter Maxi Cartridge (QIAGEN), incubated for 10 min at RT and filtered by inserting the plunger. 10 ml of the Quantum Prep Matrix was added to the cleared lysate. The matrix was pelleted by centrifugation at 3,000 x g for 5 min, the supernatant was decanted and the pellet was washed with 25ml of Wash Buffer. The matrix was resuspended in 15ml of Wash Buffer, transferred into a 25ml spin basket sitting in a 50ml Falcon tube and washed twice with Wash Buffer by centrifugation at 3,000 x g for 5 min. Finally, the DNA was eluted in 5ml of RNAse/DNAse-free water (Promega Corporation) and quantified (section II.2.14).

II.2.10 Ethanol precipitation of DNA

Two volumes of 100% cold ethanol and 0.1 volume of 3M sodium acetate pH 5.2 were added to the DNA and left on dry ice or at –70°C for at least 30 min. After centrifugation (10,000 x g, 30 min, 4°C) the supernatant was decanted. The pellet was washed with 70% ethanol and spun again at 10,000 x g for 15 min at 4°C. Supernatant was removed and the DNA pellet was air-dried prior to resuspending in either nuclease-free water (Promega Corporation) and incubated for 5 min at 65°C.

II.2.11 DNA preparation for pronuclear microinjection

Linearised DNA fragments were purified by gel extraction (section II.2.5), ethanol precipitation (section II.2.10) and resuspended in injection buffer at a concentration of about 10μg/ml. 0.5μl of the resuspended DNA fragments were run in a 0.8% agarose gel (section II.2.4) together with the 1kb DNA ladder (0.25-10 kb, Promega Corporation) and quantification standards (15-500ng, Gibco) to more accurately estimate the concentration of the fragments.

II.2.12 Extraction of genomic DNA from mice tails

Extraction of genomic DNA from mice tails was done using QIAGEN's DNeasy Tissue Kit, according to the manufacturer's instructions. Briefly, 180μl of Buffer ATL and 20μl of Proteinase K (supplied) were added to 0.5cm of tail and the tissue lysed at 55°C on a rocking platform overnight. 400μl of Buffer AL-ethanol were added and the mixture was vortexed. Tissue debris was spun down and the supernatant transferred into a DNeasy mini column. After centrifugation for 1 min at 6,000 x g, flow-through was discarded and the column washed with 500μl Buffer AW1 and subsequently with 500μl Buffer AW2. After drying the DNeasy membrane by 3 min centrifugation at 12,000 x g and incubating for 10 min at 37°C,

DNA was eluted with 200µl of 37°C warm RNAse/DNAse-free water (Promega Corporation).

II.2.13 Preparation of total RNA

Extraction of total RNA from cells was done using QIAGEN's RNeasy Mini Kit, according to the manufacturer's instructions. Briefly, cells were resuspended in an appropriate volume of the supplied RLT lysis buffer, and the samples homogenised using QIAGEN's QIAshredder spin columns. One volume of 70% ethanol was added to the homogenised lysate prior to its transfer onto a RNeasy spin column. After 15 sec of centrifugation at 8,000 x g at RT, the spin column was washed with the supplied RW1 buffer, and any contaminating DNA was digested and washed out using QIAGEN's RNAse-Free DNase according to the manufacturer's instructions. The spin column was washed again twice in the supplied RPE buffer. Finally, the RNA was eluted in 20-30µl of RNAse/DNAse-free water (Promega Corporation), quantified and its quality tested by running a RNA gel (II.2.15) or by RT-PCR (II.2.16). DNA contamination was assessed as described in section II.2.19.

II.2.14 Quantification of nucleic acids

To determine the concentration of nucleic acids in solution, absorbance at 260 and 280nm was measured in a spectrophotometer (Ultrospec 200, Pharmacia Biotech).

The readings were converted to concentrations using the following formulae (Sambrook *et al.*, 1989).

1 A_{260} unit of double-stranded deoxy ribonucleic acid (dsDNA) = 50µg/ml

1 A_{260} unit of oligonucleotide = 20µg/ml

1 A_{260} unit of RNA = 40µg/ml

To determine the purity of solutions of DNA and RNA, the ratio of absorbance at 260nm and 280nm ($OD_{260/280}$) was measured. This value should ideally be about 1.8 for DNA and 2.0 for RNA.

II.2.15 Testing RNA quality by running a RNA gel

To visualise the quality of RNA, 1µg RNA in a volume of 10µl were mixed with 2µl 6x PCR loading buffer, heated at 65°C for 5 min and cooled on ice for 3 min. RNA was run on a 1% agarose gel as described in section II.2.4.

II.2.16 Testing RNA quality by RT-PCR

In many situations, the amount of RNA is very limited making the running of a RNA gel impossible. Therefore, another method requiring less RNA was established to ensure RNA quality. The primers for β-Actin were able to amplify products in a one-tube RT-PCR reaction from both intact and partially degraded RNA (Fig. II.1B). This was probably due to the fact that the β-Actin primers amplify a relatively small product (153 base pairs (bp)), which may still be intact in partially degraded RNA. The primers for HPRT amplify a larger product (366 bp). Additionally, they span an intron and thereby ensure that only RNA and not DNA, which is less sensitive to degradation, is amplified during a one tube RT-PCR. The band intensity of the amplified HPRT product decreased substantially if partially degraded RNA was used as a starting product (Fig. II.1C). Therefore, a RT-PCR (described in II.3.19) using the primers for HPRT was carried out instead of a RNA gel to test for quality of RNA in cases where the amount of RNA was limited.

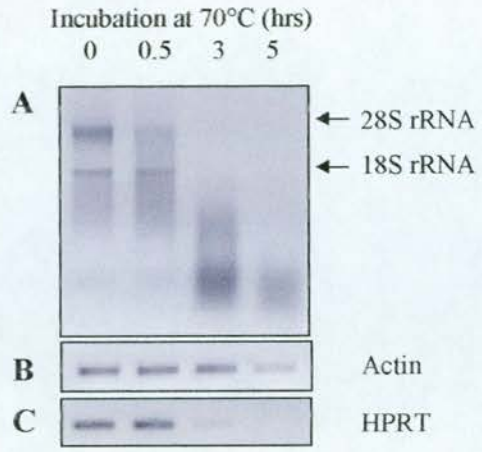


Figure II.1. Tests for RNA quality.
I-A^{b+} L cells were detached with Trypsin/EDTA and total RNA was extracted. RNA was incubated at 70°C for 0, 0.5, 3 and 5 hours.
[A] 1µg of RNA was run in a 1% agarose gel.
[B] & [C] The RNA was reverse transcribed and amplified for 25 cycles by RT-PCR. The PCR-amplified products for β-Actin [B] and HPRT [C] were run in a 2% agarose gel.

II.2.17 Primers

DNA sequences of interest were obtained from PubMed's DNA sequence programme (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). All PCR primers were designed with the following requirements: (1) not over 40-60% GC (problems with high annealing temperatures); (2) must not form primer dimers (no possibility of more than three consecutive bases on primers complementing and pairing with each other); (3) annealing temperatures (T_m) of each pair of primers must not be too different ($\Delta T_m \leq$ 4-5°C); (4) at least 16 bases, optimum 20-24; (5) must not fold into hairpin loops with energy less than 1.0, and have a free 3' tail; (6) last three bases should not all be G/Cs.

Primers were designed using the Wisconsin Genetics Computer Group (gcg) version 10 primer programme and tested using the DNA Strider programme.

Table II.1. Sequences of the primers used for amplifying genes and transcripts.
They are mouse-specific unless otherwise mentioned.

Gene	PRIMER SEQUENCE: Forward Primer Reverse primer	Length of PCR Product (bp)	T_m (°C)
Notch1*	5'- TGTTAATGAGTGCATCTCCAACCC -3' 5'- CATTCGTAGCCATCAATCTTGTCC -3'	638	58
Notch2*	5'- CAGAGGAATAGCAAGACGTGCAAG -3' 5'- GATGAAGAACAGGATGATGACAACAG -3'	599	58
Notch4*	5'- CTACTGCCCACAAGTAGCTGG -3' 5'- CTCGGAGATAGCAGTGACTGG -3'	183	60
Jagged1*[$]	5'- GGGGGTCACTGTCAGAATGA -3' 5'- AGATATACCGCACCCCTTCAG -3'	289	58
Human Jagged1	5'- CGTTGCCCACTTTGAGTATC -3' 5'- GAACAGTCATCAATGTTTGTAGAG -3'	562	59
Jagged2*	5'- ATCTGCGAGGACCTGGTGGAT -3' 5'- TATACCAGAGGGTGCGACA -3'	282	58
Delta1*	5'- GACTCTCCCGATGACCTC -3' 5'- GATGCACTCATCGCAGTAG -3'	389	60
Delta1, intron-spanning	5'- TCAGTGAGAGGCATATGGAGAG -3' 5'- TCAGTCGCTATAACACACTCATCC -3'	571 / §	56
Hes1	5'- AATGGAGAAAAATTCCTCCTCC -3' 5'- TCACCTCGTTCATGCACTCG -3'	350	56
HPRT	5'- GCTGACCTGCTGGATTACAT -3' 5'- CATTATAGTCAAGGGCATATCC -3'c	366	58
ß-Actin[$]	5'- CCACCAACTGGGACGACATG -3' 5'- GTCTCAAACATGATCTGGGTCATC -3'	153	58
EGFP[¥]	5'- ACGGCAAGCTGACCCTGAA -3' 5'- GGGTGCTCAGGTAGTGGT -3'	494	59
rtTA[¥]	5'- TTAACAACCCGTAAACTCGCCC-3' 5'- CGCAACCTAAAGTAAAATGCCCC -3'	363	60

* Specificity was confirmed by cycle sequencing of the PCR product
[$] Crossreactive with the human gene
[¥] EGFP and rtTA are engineered proteins. EGFP originates from the jellyfish *Aequorea victoria* and rtTA is a fusion protein between proteins from *E. coli* and herpes simplex virus.
§ Length of PCR product including the intron is unknown (see VI.3)

II.2.18 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was set up according to the manufacturer's instructions (Promega Corporation). Briefly, all reagents with exception of the Taq polymerase were mixed together and heated at 95°C for 10 min ("hot start") to denature the DNA. After addition of 0.5µl of the Taq polymerase, the PCR was carried out.

Each reaction tube consisted of 50µl of the following reagents:

Reagent	Volume/Tube (µl)	Final Concentration
nuclease-free water	37.0	
10x Taq Buffer with $MgCl_2$ (Buffer A)	5.0	1x
20mM dNTP mix	0.5	500µM of each dNTP
50 pM forward primer	1.0	1pM
50 pM reverse primer	1.0	1pM
~10ng plasmid or genomic DNA	5.0	200µg/µl
Taq Polymerase (5U/µl, Storage Buffer A)*	0.5	0.1U/µl

*Added after the "hot start"

RNAse/DNAse-free water (Promega Corporation) was included as a negative control for each gene tested, to ensure that there was no DNA contamination in the reagents.

The thermocycler (Pelter Thermal Cycler (PTC)-200, MJ Research) was programmed as followed:

1 cycle	94°C	10 min ("hot start")
30 cycles	94°C	1 min (denaturation)
	primer-specific annealing temperature	1 min
	72°C	1 min (amplification)
1 cycle	72°C	7 min

II.2.19 One-tube reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was reverse transcribed into cDNA and amplified by PCR using the Promega Access RT-PCR kit (Promega Corporation). The reaction was set up according to the manufacturer's instructions.

Prior to studying expression of specific genes, different RNA samples were adjusted to equal concentrations, by performing a RT-PCR on the house keeping genes, β-Actin and/or HPRT. To ensure that the measurement was performed in the exponential phase of PCR amplification, the PCR cycle number was set at 25 instead of 35 for β-Actin and HPRT.

Each reaction tube consisted of 25µl of the following reagents:

Reagent	Volume/Tube (µl)	Final Concentration
5x Reaction Buffer	5.0	1x
25mM Magnesium Sulphate ($MgSO_4$)	1.0	1mM
dNTP mix	0.5	200µM of each dNTP
Primer mix	1.0	25pmol of each primer
Avian Myeloblastosis Virus (*AMV*) RT (5U/µl)	0.5	0.1U/µl
Thermus flavus (*Tfl*) DNA polymerase (5 U/µl)	0.5	0.1U/µl
25ng RNA	16.5	1ng/ml

To test for DNA contamination in the RNA samples, the reaction was set up with all reagents except the AMV reverse transcriptase.

RNAse/DNAse-free water (Promega Corporation) was included as a negative control for each gene tested, to ensure there was no DNA contamination in the reagents.

The thermocycler (Pelter Thermal Cycler (PTC)-200, MJ Research) was programmed as follows:

- First strand synthesis:

1 cycle	48°C	45 min (reverse transcription)
1 cycle	94°C	2 min (RT inactivation and RNA/cDNA/primer denaturation)

- Second strand cDNA synthesis and PCR amplification:

35 cycles	94°C	30 secs (denaturation)
	primer-specific annealing temperature	1 min
	68°C	2 min (amplification)
1 cycle	68°C	7 min

II.2.20 Reverse transcription for real-time PCR

RNA was extracted using RNeasy kits as described above. cDNA was synthesised using the MultiScribe RT kit (PE Biosystems). The following reaction mix with a final volume of 10µl was made up, vortexed and then centrifuged.

Component	Volume/Tube (µl)	Final Concentration
10x TaqMan RT buffer	1.00	1x
25mM magnesium chloride ($MgCl_2$)	2.20	5.5mM
dNTP mix	2.00	500µM of each dNTP
Random hexamer	0.50	2.5µM
RNase inhibitor	0.20	0.4U/µl
MultiScribe RT (50U/µl)	0.25	1.25U/µl
200ng RNA	3.85	20ng/µl

The thermocycler (Perkin Elmer) was programmed as follows:

1 cycle	25°C	10 min (incubation)
1 cycle	48°C	45 min (reverse transcription)
1 cycle	95°C	5 min (RT inactivation)

After reverse transcription the samples were diluted 5:1 in RNase/DNase-free water (Promega Corporation) before proceeding to the real-time PCR step.

II.2.21 Real-time PCR

Higuchi *et al.* pioneered the analysis of PCR kinetics by constructing a system that detects PCR products as they accumulate in 'real time' (Higuchi *et al.*, 1992). Two chemistries are available to detect PCR product accumulation. One is the use of a DNA-binding dye, which has the disadvantage of binding to all double-stranded DNA, including non-specific reaction products.

Here, we used the Taqman technology (PE Systems), which exploits the 5'-3' nucleolytic activity of AmpliTaq DNA polymerase first described by Holland and colleagues (Holland *et*

al., 1991). The method uses a dual-labelled fluorogenic hybridisation probe that specifically anneals the template between the unlabelled PCR primers (Fig. II.2). The probe contains a fluorescent reporter, FAM (6-carboxyfluorescein) or VIC™, at the 5'end and a fluorescent quencher, TAMRA (6-carboxytetramethylrhodamine), at the 3'end. When the probe is intact, the emission of the reporter is suppressed by the quencher. The nuclease degradation of the hybridisation probe releases the reporter, resulting in an increase in fluorescence emission. The use of a sequence detector (ABI Prism 7700) allows measurement of the amplified product in direct proportion to the increase in fluorescence emission continuously during the PCR amplification (therefore "real-time PCR"). The amplification plot is examined early in the reaction at a point that represents the logarithmic phase of product accumulation. The point representing the detection threshold of the increase in the fluorescent signal associated with the exponential growth of the PCR product for the sequence detector is defined as the cycle threshold (C_T) (Fig. II.3). C_T values are predictive of the quantity of input target: the larger the starting concentration of a template, the lower the C_T.

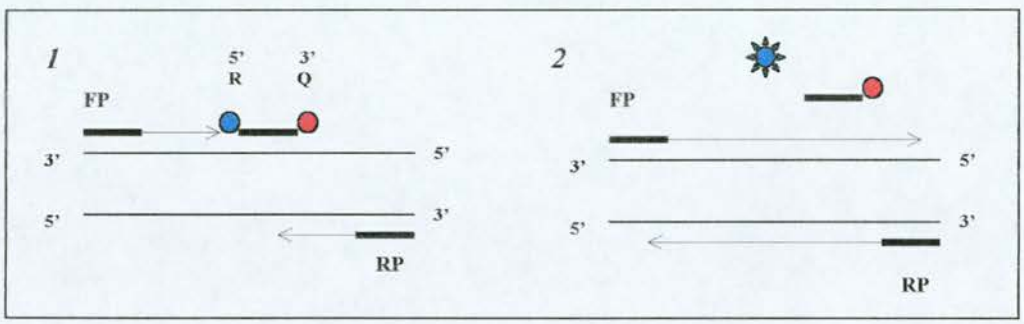


Figure II.2. Fluorogenic 5' nuclease chemistry.
PE Biosystems makes use of fluorogenic probes, which incorporate a reporter fluorescent dye (FAM or VIC) at the 5' end (R), and a quencher dye (TAMRA) at the 3' end (Q). *1.* The probe anneals downstream from one of the primer sites on the target sequence. During PCR amplification, as the primer is extended by *Taq* DNA polymerase, the probe is cleaved at its 5' end and removed from the target sequence allowing primer extension to proceed. *2.* Cleavage of the probe separates the reporter dye from the quencher dye, and the reporter emits its characteristic fluorescence. Fluorescence intensity increases proportional to the amount of PCR product amplified.
Diagram adapted from PE Biosystems.

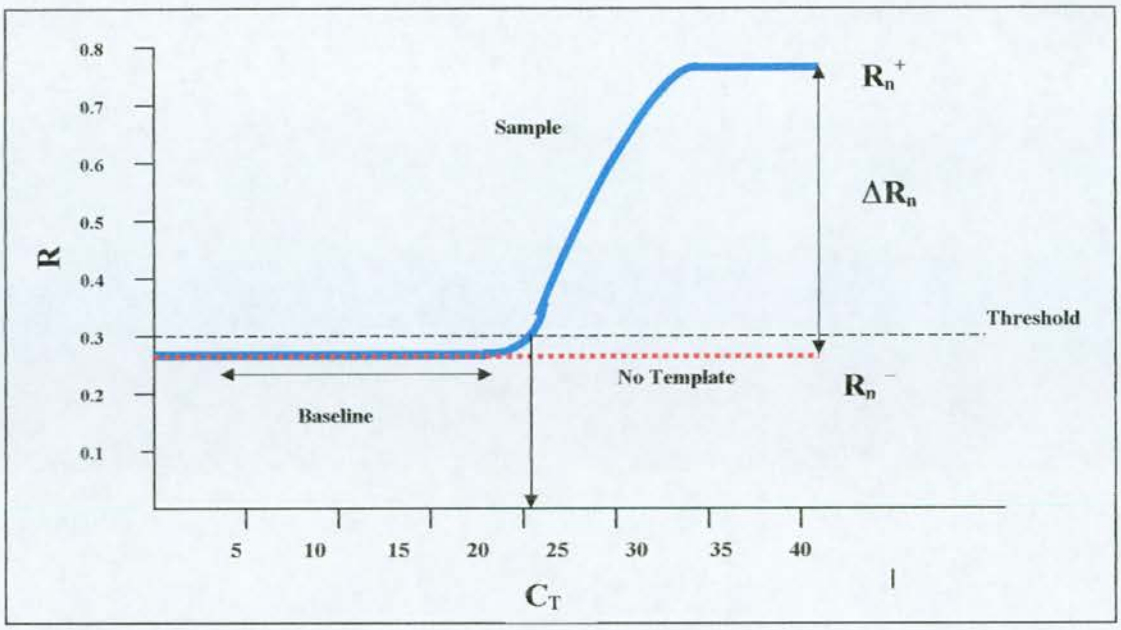


Figure II.3. Typical amplification plot.
An amplification plot is the plot of fluorescence signal versus cycle number. In the initial cycles of PCR there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated product. A fixed fluorescence threshold can be set above the baseline.

- C_T (threshold cycle) is defined as the cycle number at which the fluorescence passes the fixed threshold.
- R_n is the value obtained by dividing the emission intensity of each reporter dye signal with that of an internal reference dye (ROX), which normalises for non-PCR related fluorescence fluctuations occurring well-to-well or over time.
- R_n^+ is the R_n value of a reaction containing all components including the template.
- R_n^- is the R_n value obtained from either the early cycles of a real-time run (prior to detectable increase in fluorescence) or from a reaction not containing template.
- ΔR_n is the difference between the R_n^+ and the R_n^- value. It reliably indicates the magnitude of the signal generated by the given set of conditions.

Diagram adapted from PE Biosystems.

II.2.21.1 Real-time PCR optimisation

The following parameters need to be optimised for efficient real-time amplification of target cDNA:

A) Primer and Probe Design and Optimisation

Primers and probes are designed using Primer Express® software, supplied by PE Biosystems.

The primers are optimised by independently varying forward and reverse primer concentrations (50, 300, 900nM). Optimal performance is achieved by the primer concentrations that provide the lowest C_T and the highest ΔR_n.

The probe can also be optimised by varying the concentration from 50 to 250nM. Optimal performance is obtained at the concentration that provides the lowest C_T and the highest ΔR_n. However, a probe limiting concentration should be avoided when wishing to detect low copy numbers of a target sequence.

In real-time PCR assays examined here, primer concentrations of 300nM for both the forward and reverse primer, and probe concentrations of 200nM per reaction, were used.

B) The Comparative Method of Quantitation

Relative quantitation of gene expression can be performed using the comparative ($\Delta\Delta C_T$) method. This method needs:

- A **target**, which is the sample to be tested.
- A **reference**, which can be an endogenous control with its own set of primers and probe. It is used to standardise all the cDNA samples used in a reaction, and as a control of the PCR reaction itself.
- A **calibrator**, this is the positive control used in the reaction, which all the targets are made relative to. Thus, the calibrator becomes the 1x sample, and all other quantities are expressed as an *n*-fold difference relative to the calibrator.

The arithmetic formulas used for the comparative method:

The amount of target, normalised to an endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta C_T}$, where

- ΔC_T is the C_T of the gene of interest – C_T of the endogenous control (normalises the amount of target to the endogenous control).
- $\Delta\Delta C_T$ is the ΔC_T of the target – ΔC_T of the calibrator (makes the amount of target relative to the calibrator).

For the $\Delta\Delta C_T$ to be valid, the efficiency of the target amplification and of the reference amplification must be approximately equal. This can be assessed by running dilutions of a stock cDNA and plotting the standard curves for the gene of interest and for the endogenous control.

II.2.21.2 Multiplex PCR

Multiplex PCR uses more than one mix of primer pair and probe in the same tube. This method can be used in relative quantitation where one primer pair amplifies the target and another primer pair amplifies the endogenous reference (e.g. a housekeeping gene) in the same tube.

For accurate quantitation it is important that the two reactions do not compete. This can be avoided by limiting the concentration of primers of the most abundant species. The desired concentration is that which shows a reduction in ΔR_n but little effect on C_T.

The following components were used throughout these studies:

- **Endogenous control**: PE Biosystems 18S ribosomal RNA (rRNA). This is already primer limited to avoid competition for the amplification of the target sample. It contains specific primers and a probe that is labelled with the fluorogenic dye VIC.
- **Universal calibrator**: This is a sample that contains transcripts of all the genes analysed and was used as a control for the run of each individual plate. The universal calibrator for murine genes contained cDNA from thymocytes and PMA/ionomycin-, ConA-activated and non-activated splenocytes of different mice strains (BALB/c, C57BL/6 and F1). The universal calibrator for human Jagged1 contained cDNA from PMA/ionomycin-activated and non-activated Jurkat cells.
- **Internal calibrator**: Within each set of experiment an internal calibrator, e.g. untreated cells or untransfected cells, was chosen to show gene expression relative to the internal calibrator.
- **No template control** for each specific gene tested, to ensure that there was no DNA contaminating the samples.

II.2.21.3 Reaction set-up for real-time PCR

PCR was carried out using TaqMan Universal Master Mix (PE Biosystems) containing AmpliTaq Gold DNA polymerase, dNTPs, passive reference dye (ROX) and optimised buffer components. Pre-optimised primer/probe mix for the 18S rRNA (PE Biosystems) was included in each reaction as the endogenous control. All reactions were run in duplicates. 2.5µl of each cDNA was aliquoted into two separate wells of MicroAmp optical 96-well plate (PE Biosystems). As a positive control, cDNA of the universal calibrator (described above) was run in duplicate, as was a no-template negative control. 22.5µl of the following PCR mix were added per well to bring the total well volume to 25µl:

Reagent	Volume/Well (µl)	Final Concentration
10x Universal Master Mix	2.50	1x
20x Pre-Developed 18S rRNA	1.25	1x
Primer/Probe Mix	7.00	300nM for both primers 200nM for probe
Water	1.75	

MicroAmp optical caps were carefully pressed onto each well and the plate was placed in the ABI Prism 7700 sequence detector running SDS software (PE Biosystems) and using a heated lid. The same cycling programme (see below) was run for all primer/probe combinations.

Real-time PCR programme:	1x	50°C	2 min
	1x	95°C	10 min
	40x	95°C	15 sec
	1x	60°C	1 min

All the values obtained were normalised to 18S rRNA and made relative to the universal calibrator described above. The data was plotted relative to the internal calibrator (e.g. I-A^{b+} L cells in Fig. V.3, or control $CD4^+$ T cells in Fig. V.10).

Table II.2. Sequences of murine gene-specific primers for real-time PCR.

PRIMERS	PRIMER SEQUENCE Forward Primer (5'-3') Reverse Primer (5'-3')	PROBE
Notch1	TCCAGAGTGCCACCGATGT TCCACCGGCTCACTCTTCAC	CTGCCTTCCTAGGTGCTCTTGCGTCA
Notch2	ACCCTCCGCCGAGACTCT TCCCAGAACCAATCAGGTTAGC	CCTGTCCCACAGGTTCACGGCG
Notch3	GGCTGCAACACTGAGGAATGT TCAGGAGGCAGAAGAACTGTGA	CCGGGACCTCGCTGGCACA
Notch4	TGTCTCCCCCATAGAGTATGCA CTCGAAATCAACTTTGTCCTCTTG	CCGGACATCCTAAACCCTCTTCCCATTG
Delta1	TTCTTTCGCGTATGCCTCAA CATCAGGCAGGCTGAAGGA	ACTACCAGGCCAGCGTGTCACCG
Delta3	GGCGGTGAAGATCCTGACTCT CGGTCCACCCTCTTCTCACA	ATTGCCCACCTGGTTTCCAAGGCTC
Jagged1	CCCGCACCCAGGATGTGT CACCCCAGTTGGTCTCACAGA	CACCTGCAATGAACCCTGGCAGTG
Jagged2	CAGCTGGACGCCAATGAGT GCCAATCAGGTTTTTGCAAGA	AGCATTAAGGCACGGCTTCCCTTCA
Deltex1	GCCTCTAGCCTGGCACATG CCAGATCCCCTTAGCGCTTCT	CTCCCTCCGCTCTCGGCGG
Hes1	GCTTCAGCGAGTGCATGAAC TTGATCTGGGTCATGCAGTTG	TGACCCGCTTCCTGTCCACGTG
Hes5	GGTTGGCCGCTACCTTCTTC TCTGCACACACATTCTCTAAGAATGAC	TCCCTCCTCCGGCTGGCTGG
IL-2	PE Biosystems Cat. No. 4311216	
IL-4	CGCCATGCACGGAGATG ACGAGCTCACTCTCTGTGGTGTT	TGCCAAACGTCCTCACAGCAACGA
IL-10	CCACAAAGCAGCCTTGCA AGTAAGAGCAGGCAGCATAGCA	AGAGCTCCATCATGCCTGGCTCAGC
IL-12p35	GTGAAAATGAAGCTCTGCATCCT TCAGGCGGAGCTCAGATAGC	CACGCCTTCAGCACCCGCG
IL-12p40	ACATCTACCGAAGTCCAATGCA GGAACACATGCCCACTTGCT	CGTGCAAGCTCAGGATCGCTATTACAATTC
IFNγ	CCTGCGGCCTAGCTCTGA GCCATGAGGAAGAGCTGCAA	ACAATGAACGCTACACACTGCATCTTGGC

Table II.3. Sequences of human gene-specific primers for real-time PCR.

PRIMERS	PRIMER SEQUENCE Forward Primer (5'-3') Reverse Primer (5'-3')	PROBE
Jagged1	CTTAACTGTGGCTTGGATCTGTTG GTCCTCAGAGGCTGAGTGTGTGT	CTGCCTTCCTAGGTGCTCTTGCGTCA

II.2.21.4 Significance of real-time PCR

PCR reactions were run in duplicates. Therefore, standard deviation cannot be assessed. However, Martell and colleagues reported a high reproducibility within a real-time PCR run (intra-assay) or between different PCR runs (inter-assay) (Martell *et al.*, 1999). Further, genes were considered as differentially expressed if a change of at least twofold or greater was observed in at least two separate experiments (Chtanova *et al.*, 2001; Granucci *et al.*, 2001). However, both groups used oligonucleotide microarrays for expression analysis. Generally, I considered a change in expression of at least 3-fold or greater on at least two occasions as significant because I observed that smaller changes were often not reproducible. Exceptions to this rule were made if the difference in expression on RNA level was reflected on protein level or if an alteration of a treatment, which induces strong upregulation of gene expression, leads to a reproducible change in transcript expression compared to the original treatment.

II.2.22 Cycle sequencing of PCR products isolated from agarose gels

Cycle sequencing (also called linear amplification sequencing) is performed by cycling the PCR products through repeated rounds of denaturation, annealing and extension in a temperature cycler (Murray, 1989; Sanger *et al.*, 1977). cDNA was sequenced using the Cyclist Taq DNA Sequencing kit (Stratagene). The PCR products were isolated from agarose gels as described in section II.2.5.

II.2.22.1 Primer end labelling

The forward primer was labelled with [γ-^{33}P]ATP by incubating 0.1µg primer with 1µl T4 polynucleotide kinase (PNK, 10U/µl, Stratagene), 2.5µl 10x PNK buffer (Stratagene), 6µl [γ-^{33}P]ATP (10mCi/ml, Pharmacia Biotech) and DEPC-treated water at a final volume of 25µl at 37°C for 45 min.

II.2.22.2 Sequencing reaction

The sequencing mix was prepared by adding 10µl of purified PCR product to 1µl (2U/µl) Taq DNA polymerase (supplied), 4µl 10x sequencing buffer containing all four deoxyribonucleoside triphosphates (dNTPs) (supplied), 4µl labelled primer and adding DEPC-treated water to a final volume of 30µl. 6.7µl were added to each of the four termination tubes already containing 3µl of ddNTP (supplied) and kept on ice. As the ddNTP lacks the necessary 3'-OH group required for chain elongation, the growing oligonucleotide was terminated selectively at A, C, G or T, depending on the respective dideoxy analog in the reaction (Sanger *et al.*, 1977). The relative concentrations of dNTPs and ddNTPs were balanced so that the majority of chains will be terminated within the desired length of sequence.

The thermocycler (PTC-200, MJ Research) was programmed as follows:

35 cycles	95°C	20 sec (denaturation)
	specific primer annealing temperature (T_m)	20 sec (annealing)
	72°C	20 sec (amplification)

The reaction was stopped by addition of 5µl stop solution (supplied) to each termination tube. The fragments were separated on a sequencing gel, and sequence information was obtained from the order of the bands.

II.2.22.3 Sequencing gel

To prepare the 6% denaturing polyacrylamide gel, 33.6g urea were dissolved in 8ml 10x TBE (Gibco), 16ml 30% (w/v) acrylamide-bisacrylamide solution (Kramel Biotech), and 29ml of distilled water (to give a final volume of 80ml), by magnetic stirring and slight heating.
150µl of 10% APS (ammonium peroxidisulphate) and 150µl of TEMED (N,N,N',N'-tetramethylethylenediamine) (Promega Corporation) were then added to initialise polymerisation. The gel solution was poured into prepared glass plates.
Once the gel had set, it was pre-run for 30 min at 65 watts. The samples were denatured at 95°C for 7 min and loaded into the wells. The gel was run for 2 hrs in 1x TBE buffer. The gel was transferred to filter paper (Whatmann), dried using a gel drier (Bio-Rad) for 1 hour at 80°C, and exposed for 24 hrs or appropriate time onto an autoradiograph film (Biomax MR-1, Kodak).

II.3 Cell Biology Techniques

II.3.1 Culturing of bone marrow (BM)-derived dendritic cells (DCs)

II.3.1.1 Bone marrow (BM) preparation

Femurs and tibiae of BALB/c or C57BL/6 mice were cleaned from the surrounding muscle tissue by rubbing with Bacterial Disinfectant wipes (Premier). Intact bones were dipped into 70% ethanol for disinfection and washed with PBS. Then both ends were cut with scissors and the marrow flushed out with complete RPMI using a syringe with a 0.45mm (26-gauge) diameter needle. Clusters within the marrow suspension were disintegrated by vigorous pipetting and cells pelletted by centrifugation for 7 min at 300 x g. The pellet was resuspended in 1ml of cold Gey's red blood cell (RBC) lysis buffer and left at RT for 1 minute. Cell suspension was then diluted down in complete RPMI to 10ml and washed as before. The pellet was resuspended in 5ml of complete RPMI, and cells were counted. Typically 30-40x10^6 cells were obtained per mouse.

II.3.1.2 Generation of BM-derived DCs based on Scheicher's protocol

The current protocol was adapted from previous publications (Lutz *et al.*, 1999; Scheicher *et al.*, 1992). At day 0, BM cells were cultured in 10ml of complete Iscoves supplemented with 20ng/ml recombinant murine GM-CSF (rmGM-CSF) (R&D Systems) in bacteriological petri dishes (Greiner) with 100mm diameter at a concentration of 2x10^5 cells/ml. Bacterial petri dishes were used because more DC were produced than on tissue culture plastic. The modified cell adherence condition of the plastic appears to hamper the development of macrophages, and thus the development of DCs in suspension might be favoured (Lutz *et al.*, 1999). At day 3, another 10ml of complete Iscoves containing 20ng/ml rmGM-CSF were added to the plates. At days 6 and 8, half of the culture supernatant was collected, centrifuged, and the cell pellet resuspended in 10ml fresh complete Iscoves containing 10ng/ml rmGM-CSF, and added back to the original petri dish. The majority of co-evolving granulocytes (and also contaminating B

cells) died off by day 10, especially by reducing the doses of GM-CSF from day 6 onwards (Lutz *et al.*, 1999). At day 10, non-adherent cells were collected by gentle pipetting, centrifuged at 300 x g for 7 min at RT and resuspended in 10ml fresh complete Iscoves at $2.5x10^5$ cells/ml into a new 100mm petri dish containing 10ng/ml rmGM-CSF and 50ng/ml murine TNFα (R&D Systems) ± 50ng/ml IL-10 (R&D Systems). Cells were then cultured for another 64 hrs.

Expected yield: 150% of starting cell number at day 10 of culture and 50% after maturation for additional two days. Over 95% of the non-adherent cells had a classical DC morphology on day 12 of culture (E. Jarman, unpublished data).

II.3.1.3 Generation of BM-derived DCs based on Inaba's protocol

Following a protocol based on Inaba's method (Inaba *et al.*, 1992a), $3.75x10^5$ cells/well were placed into 24-well plates in 1ml of DC-RPMI supplemented with 5% GM-CSF supernatant (see II.3.1.5). The cultures were fed at day 3 and 6 by gently swirling the plates, aspirating 80% of the medium and adding back fresh DC-RPMI with 5% GM-CSF. These washes removed the majority of non-adherent granulocytes and B cells without dislodging clusters of developing DCs that were loosely attached to firmly adherent macrophages. At day 7, aggregates of attached cells were dislodged by pipetting cells and plated at 10^6 cells/ml/well into a new 24-well plate for maturation with DC-RPMI containing 5% GM-CSF and 50ng/ml murine TNFα (R&D Systems) ± 50ng/ml IL-10 (R&D Systems) or 100ng/ml LPS (SIGMA). Cells were then cultured for up to 72 hrs.

Expected yield: 100-150% of starting cell number at day 7 of culture and 70% and 50% after maturation with TNFα or LPS, respectively, for additional two days.

II.3.1.4 Enrichment of DCs using paramagnetic beads

Enrichment of DCs was effected by depleting contaminating granulocytes using paramagnetic beads (Dynabeads M-450) coated with antibodies specific for rat IgG isotypes (Dynal). These were used in combination with other antibodies raised in rats specific for appropriate cell surface markers (e.g. Gr1).

Contaminating granulocytes of the DC culture ($3-5x10^6$ total cells) were coated with 10μg/ml anti-Gr1 antibody (clone RB6-8C5, Pharmingen) in a volume of 500μl complete RPMI for 30 min at 4°C on a rotating mixer (Stuart Scientific). Excess antibodies were removed by

washing twice with cold complete RPMI. Prior to use, the beads were washed several times with fresh PBS to remove azide and finally resuspended in 250µl of complete medium per $2.5x10^6$ beads. Coated cells ($3-5x10^6$) were resuspended in 500µl of the bead suspension and incubated for 30 min at 4°C on a rotating mixer. Beads were then collected using a strong magnet (MPC-S, Dynal) and the supernatant containing the unbound cells removed and retained for further processing.

II.3.1.5 GM-CSF supernatant

The GM-CSF supernatant was a kind gift of G. Perona-Wright. It was produced by transfected hybridoma (X63-gmcsf), which was a kind gift of D. Gray (ICAPB, University of Edinburgh; Stockinger *et al.*, 1996). It contains 200-300ng/ml GM-CSF, 1.7ng/ml IL-10 (10-15ng/ml and 90pg/ml if used at 5%, respectively), but no detectable levels of TNFα or IL-12 (L. Stuart, personal communication).

II.3.2 Preparation of splenocyte suspension

Spleens were removed from C57BL/6 ($H-2^b$) or BALB/c ($H-2^d$) female mice, aged 6-8 weeks. The spleens were placed in a 70µm cell-strainer (Becton Dickinson) sitting on a 6cm petri dish containing 5ml of PBS. The tissues were ground using the end of a 1ml syringe plunger. Alternatively, tissues were ground between 2 glass slides until a homogenous cell suspension was obtained. Cell suspensions were passed through 30µm pre-separation filters (Miltenyi Biotech) and washed by centrifuging at 300 x g for 7 min at RT. The supernatant was discarded and the pellet was resuspended in 2ml of cold Gey's red blood cell (RBC) lysis buffer per spleen and left at RT for 1 minute. Cell suspensions were then diluted down in complete RPMI to 15ml and washed as before. The pellet was resuspended in 10ml of PBS, and the spleen cells were counted. Typically $80-120x10^6$ cells were obtained per spleen.

For removal of dead cells, the spleen suspension were spun down, diluted in 2ml of RT complete RPMI and gently layered over 2ml of RT lympholyte-M (Cedarlane, U.K.) per spleen, and centrifuged at 1.500 x g, RT for 25 min. The interface was carefully removed and washed 2 times with complete RPMI. The viable cells were counted using a haemacytometer with 0.4% trypan blue exclusion. Cells were usually over 95% viable.

II.3.3 Cell isolations by MACS (Magnetic Cell Sorting)

Cell populations were isolated from the spleens of BALB/c or C57BL/6 mice using a magnetic cell sorting (MACS) method (Miltenyi Biotech). This technique relies on antibodies conjugated to magnetic microbeads that recognise cell-specific surface markers, which will bind to a magnetic column when placed in a magnetic field. Therefore, cells expressing cell surface proteins that are recognised by the antibodies will bind to the column and can subsequently be eluted as a highly pure population. All other cell types are collected in the negative fraction.

The following microbead-conjugated antibodies were used:

- anti-CD4 (LT34) microbeads-antibody for separation of $CD4^+$ T cells.
- anti-CD8 (Ly-2) microbeads-antibody for the separation of $CD8^+$ T cells.

For separation of $CD4^+$ or $CD8^+$ T cells, spleens were processed as described in section II.3.2. After cell counting, the spleen suspension was washed with cold MACS buffer and the pellet was resuspended at a concentration of 900µl/10^8 total cells in cold MACS buffer. 100µl/10^8 cells of specific microbead-conjugated antibody were added, the cell suspension was mixed and incubated for 15 min at 4°C. The cells were washed and the pellet resuspended in 500µl of cold MACS buffer before loading onto a LS MACS column sitting on a VarioMACS separator. The column was washed with 3x 3ml cold MACS buffer and the positive fraction eluted with 5ml cold MACS buffer using the supplied plunger. The cells were counted. A fraction was kept for measuring cell purity by flow cytometry. The rest was kept for use in proliferation assays or RNA extraction. Purities of MACS-isolated $CD4^+$ and $CD8^+$ T cells were 95±2% and 88±6%, respectively (Fig. II.4).

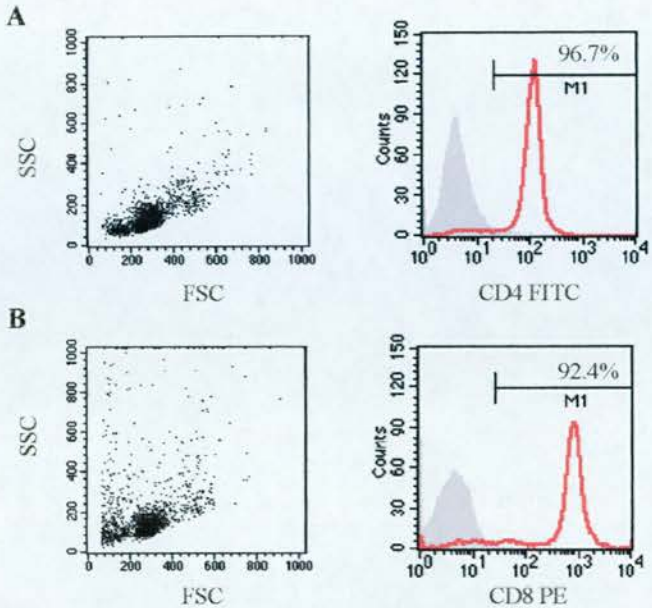


Figure II.4. Purity of MACS-purified $CD4^+$ and $CD8^+$ T cells.
$CD4^+$ or $CD8^+$ T cells were purified by MACS from lympholyte-treated BALB/c spleens, stained with anti-CD4 or anti-CD8 antibodies, respectively, and purity assessed by flow cytometry. Forward scatter (FSC) and side scatter (SSC) analysis and histogram of ungated MACS-purified $CD4^+$ and $CD8^+$ T cells ([A] and [B], respectively). Shaded histogram: Isotype control, thick red line: indicated antibody. Numbers within the histograms represent percentages within the marker bounds. Markers were set using isotype control stainings. Details of antibodies are given in table II.4 & II.5.

II.3.4 Purification of $CD4^+$ T cells by depletion using R&D columns

R&D depletion columns were used according to manufacturer's instruction to isolate highly enriched $CD4^+$ T cells via high affinity negative selection. Briefly, 10^8 splenocytes resuspended in 1ml of 1x Column Buffer (R&D Systems) were mixed with 1ml of monoclonal antibody cocktail (R&D Systems) and incubated for 15 min at RT. The cells were washed twice before being resuspended in 2ml of 1x Column Buffer and applied onto the column (R&D Systems), which had been rinsed previously with 10ml of 1x Column Buffer. After a 10-min incubation at RT, unbound cells were eluted from the column with 10ml of 1x Column Buffer and washed once with complete RPMI. Purities of depleted $CD4^+$ T cells were 84-91% (R&D Systems).

II.3.5 Enrichment of T cells by depletion using paramagnetic beads

Negative depletion was used in order to isolate T cell populations from splenocyte suspension following a similar protocol already described for enrichment of DCs (II.3.1.4). Briefly, splenocytes were coated with 10µg/ml anti-B220, 10µg/ml anti-I-A^d/I-E^d and 10µg/ml anti-Gr1 monoclonal rat antibodies (clones RA3-6B2, 2G9 and RB6-8C5, respectively, Pharmingen) in 1ml of complete RPMI per $5x10^7$ splenocytes at 4°C on a rotating mixer (Stuart Scientific) for 30 min. Excess antibodies were removed by washing twice with cold complete RPMI. Prior to use, the beads were washed several times with fresh PBS to remove azide and finally resuspended in 5ml of complete medium per 10^6 beads. Coated cells ($5x10^7$) were resuspended in 5ml of the bead suspension and incubated for 30 min at 4°C on a rotating mixer. Beads were then collected using a strong magnet (MPC, Dynal) and the supernatant containing the unbound cells removed and retained for further processing.

II.3.6 Enrichment of T cells using nylon wool fibre columns

Differential adherence properties of T cells, B cells and accessory cells such as macrophages or other APCs can be employed to enrich for T cells. Nylon wool can be used as a column matrix to which B cells and accessory cells adhere more readily than T cells (Julius *et al.*, 1973). Columns containing 0.5g nylon wool fibres (Polysciences) were washed and then incubated with complete RPMI at 37°C in a humidified incubator with 5% CO_2 for 1 hour. The media was drained to the top of the nylon wool column. Lympholyte-treated splenocytes and lymph node cells were resuspended in 1.5ml/spleen warm complete RPMI, loaded onto the nylon wool column and another 2 ml of warm media was added to column to ensure that the top of the wool is covered with media. After 1 hour incubation at 37°C in a humidified incubator with 5% CO_2 the non-adherent T cells were collected by washing the column with 15-20ml warm complete RPMI. Typically $20\text{-}30x10^6$ cells were obtained for spleen and lymph nodes from one mouse consisting of around 78±8% T cells (59±5% $CD4^+$ T cells and 20±5% $CD8^+$ T cells) (Fig. II.5).

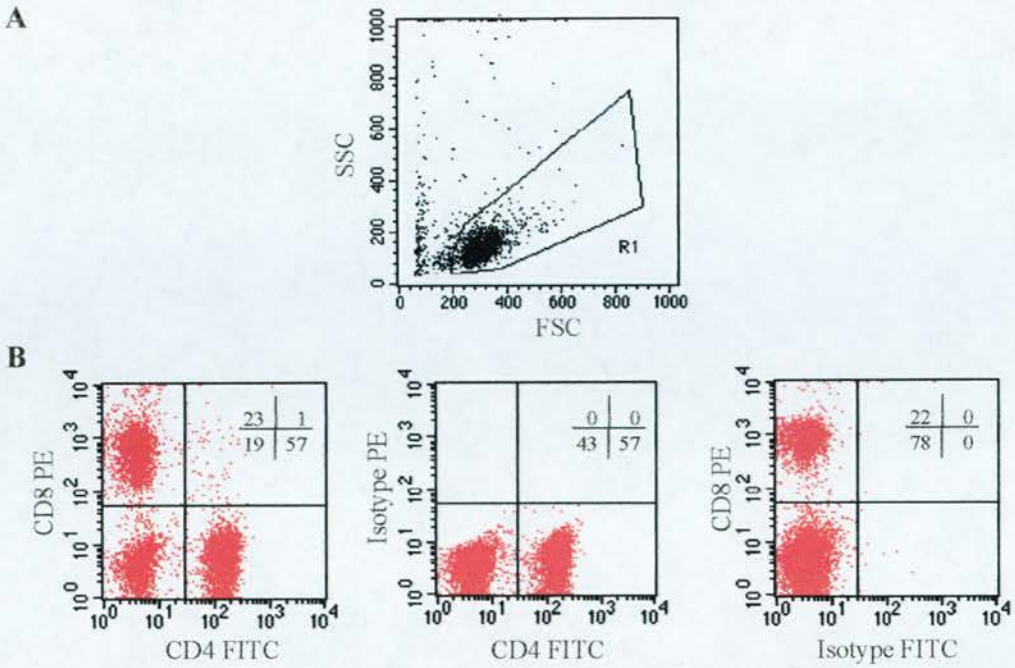


Figure II.5. Purity of nylon wool-enriched T cells.
T cells from lympholyte-treated BALB/c spleens were enriched by passing through a nylon wool column. The cells were double stained with anti-CD4 and anti-CD8 antibodies and the corresponding isotype controls and analysed by flow cytometry. Details of antibodies are given in table II.4 & II.5.
[A] Forward scatter (FSC) and side scatter (SSC) analysis.
[B] Dot plot analysis of R1-gated cells. Quadrant grid was set using isotype staining. Numbers in the cross within dot plots represent percentages of the corresponding quadrant.

II.3.7 Labelling of nylon wool-enriched T cells with CFSE

CFSE (carboxyfluorescein diacetate succinimidyl ester) is a membrane-permeate dye that covalently attaches to cytoplasmic proteins (Lyons and Parish, 1994). Approximately 99% of the initial label is metabolised within the first 24 hrs. Only cytoplasmic proteins with a relatively long half-life are still labelled at later time points. Upon division, CFSE is distributed evenly between daughter cells and the mean fluorescence halves accordingly. In this way division can be progressively analysed by flow cytometry up to 8-10 cell cycles. The limiting factor of detectable cell cycle number is the autofluorescence of the cells. Importantly, cellular differentiation is unaffected by the dye because labelled lymphocytes have been shown

to isotype switch, develop cytokine secretion potential and alter their cell surface phenotype as normal (Gett and Hodgkin, 1998; Hasbold *et al.*, 1998).

Nylon wool-enriched T cells were washed once in PBS. The cell pellet was resuspended in 2.5μM CFSE/PBS at $5x10^7$ cells/ml. After a 10-minute incubation at 37°C, the cells were washed 3 times with cold complete RPMI.

II.3.8 Flow cytometry

II.3.8.1 Analysis of cell surface molecules by flow cytometry

Flow cytometric analysis of cell surface expression of proteins was carried out in a Becton Dickinson FACSCalibur (BD Immunocytometry Systems). For direct antibody labelling, 1-$2x10^5$ cells/sample were added to flexible 96 round-bottom well plates (Becton Dickinson), and washed for 4 min at 300 x g, 4°C. The cells were pre-incubated in FACS buffer + 10% mouse serum (Scottish Antibody Production Unit-SAPU) for 15 min at 4°C to decrease non-specific binding of the antibodies. The cells were washed and resuspended in 50μl of FACS buffer + 10% mouse serum containing 0.5μg of FITC-labelled and/or 0.2μg of PE- and/or Cy-Chrome-labelled antibody. The cells were incubated in the dark at 4°C for 20 min, and washed 2 times in cold FACS buffer. Cells were resuspended in 400μl of cold FACS buffer. Fluorescence of live cells was analysed by adding 0.5μl 7-aminoactinomycin D (7-AAD) (eBioscience) prior to acquisition of the sample. Dead cells took up 7-AAD and could be separated from the live cells due to their positive fluorescence in the FL3 channel. If the cells were not analysed immediately, they were resuspended in 200μl of PBS, fixed by adding 200μl FACS fixing buffer and stored at 4°C for a maximum of 2 days. Fluorescence of fixed cells was analysed without adding 7-AAD.

Table II.4. Antibodies used for flow cytometry.

Antibody to	Clone	Isotype[§]	Fluorochrome[Ψ]	Supplier
anti-rat IgG	N/A[$]	Goat $(Fab')_2$	PE	Serotec
CD3ε	145-2C11	HsIgG, gp1, κ	PE	Pharmingen
CD4/L3T4	GK1.5	rIgG2b, κ	Cy/FITC/PE	Pharmingen
CD8α, Ly2	53-6.7	rIgG2a, κ	Cy/FITC/PE	Pharmingen
CD11c	HL3	HsIgG, gp1, λ	PE	Pharmingen
CD14	RmC5-3	rIgG1, κ	PE	Pharmingen
CD19	1D3	rIgG2a, κ	PE	Pharmingen
CD25	3C7	rIgG2b, κ	FITC/PE	Pharmingen
CD40	3/23	rIgG2a, κ	PE	Pharmingen
CD45R/B220	RA3-6B2	rIgG2a, κ	FITC	Pharmingen
CD54/ICAM-1	3E2	HsIgG, gp1, κ	PE	Pharmingen
CD69	H1.2F3	HsIgG, gp1, λ	PE	Pharmingen
CD80/B7.1	16-10A1	polyclonal HsIgG	PE	Pharmingen
CD86/B7.2	GL1	rIgG2a, κ	PE	Pharmingen
CD152/CTLA4	UC10-4F10-11	HsIgG, gp1, κ	PE	Pharmingen
CD169/Sialoadhesin	3D6.112	rIgG2a	unconjugated	Serotec
F4/80	CI:A3-1	rIgG2b	FITC	Caltag
H-2K^k	36-7-5	mIgG2a, κ	PE	Pharmingen
I-A/I-E *	2G9	rIgG2a, κ	FITC/PE	Pharmingen
IFNγ	XMG1.2	rIgG1	PE	Pharmingen
IL-4	11B11	rIgG1	PE	Pharmingen
Ly-6G (Gr1)	RB6-8C5	rIgG2b, κ	PE/unconjugated	Pharmingen
TCRβ	H57-597	HsIgG, gp2, λ	PE	Pharmingen

[§] HsIgG = hamster immunoglobulin G, rIgG1/2a/b = rat immunoglobulin 1/2a/b
[Ψ] Cy = Cy-Chrome, FITC = fluorescein isothiocyanate, PE = phycoerythrin
* Crossreactive with I-A/I-E from mice with haplotype H-2^d, H-2^b, H-2^k, H-2^p and H-2^q
[$] Not applicable

Table II.5. Isotype controls used for flow cytometry.

Controls	Clone	Isotype[§]	Fluorochrome[Ψ]	Supplier
Isotype HsIgG	N/A[$]	polyclonal	PE	Pharmingen
Isotype rIgG2a, κ	R35-95	rIgG2a, κ	Cy/FITC/PE	Pharmingen
Isotype rIgG2b, κ	A95-1	rIgG2b, κ	FITC/PE	Pharmingen
Isotype rIgG1, κ	R3-34	rIgG1, κ	PE	Pharmingen
Isotype rIgG2b	N/A[$]	rIgG2b	FITC	Caltag

[§] HsIgG = hamster immunoglobulin G, rIgG1/2a/b = rat immunoglobulin 1/2a/b
[Ψ] Cy = Cy-Chrome, FITC = fluorescein isothiocyanate, PE = phycoerythrin
[$] Not applicable

II.3.8.2 Detection of intracellular cytokines by flow cytometry

After primary stimulation of the nylon wool-enriched T cells in a mixed lymphocyte reaction (MLR) with murine fibroblast transfectants (L cells), cells were restimulated for 4 hrs by adding PMA and ionomycin to a final concentration of 50ng/ml and 500ng/ml, respectively. As a control, an aliquot of the cells received no restimulation. 667µl/ml of GolgiStop (Pharmingen) was added for the final 3 hrs. Then cells were harvested and lympholyte-M (Cedarlane) treated to remove dead cells and cell debris as described in section II.3.2. $2x10^5$ cells/sample were stained with Cy-Chrome labelled anti-CD4 or anti-CD8 antibodies described in II.3.8.1) followed by fixation with 100µl of 4% paraformaldehyde/PBS for 10 min on ice. Intracellular cytokines were detected by a modified method of Pala *et al.* (2000). Briefly, cells were permeabilised by washing with 200µl of permeabilisation buffer followed by a 10-min incubation in 200µl permeabilisation buffer at RT in the dark. After centrifugation, cells were resuspended in 50µl permeabilisation buffer containing 1µl of PE-labelled anti-IFNγ or anti-IL-4 antibody. After incubating for 30 min at RT in the dark, cells were washed twice with permeabilisation buffer and once with FACS buffer before being resuspended in 200µl FACS buffer. Samples were ready to be analysed immediately or they could be stored for up to 3 days.

II.3.8.3 FACS (fluorescence-associated cell sorting)

Cell sorting by flow cytometry was performed for nylon-purified splenic T cells, which were cultured with L cells. Dead cells were removed by lympholyte-M as described in section II.3.2. The cells were double stained with 7.5µg/10^7 cells of FITC-CD4 and PE-CD8 antibodies in 500µl/10^7 cells of HBSS + 15% FCS + 10% mouse serum, and incubated for 30 min at 4°C in the dark. The cells were washed 2 times in HBSS + 15% FCS, passed through a 30µm pre-separation filter (Miltenyi Biotech) and resuspended in HBSS + 15% FCS at $1.5x10^7$ cells/ml. Dead cells and remaining L cells were excluded by addition of 4µl/10^6 7-AAD just prior to FACS sorting (BD FACSVantage SE, BD Immunocytometry Systems). Cell sorting of enhanced green fluorescent protein (EGFP)-expressing L cells into EGFP high ($EGFP^{hi}$), EGFP low ($EGFP^{lo}$) and EGFP negative cells, cells were passed through a 30µm pre-separation filter and resuspended in HAT+MX medium at $5x10^6$ cells/ml ready for FACS sorting.

II.3.9 Apoptosis assay (Annexin V staining)

In the early stages of apoptosis phosphatidylserine (PS) translocates from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell. Annexin V is a Ca^{2+} dependent phospholipid-binding protein with high affinity for PS. Hence this protein can be used as a sensitive probe for PS exposure on the cell surface. Translocation of PS to the external cell surface is not unique to apoptosis, but occurs also during cell necrosis. The difference between these two forms of cell death is that during the initial stages of apoptosis the cell membrane remains intact, while at the very moment that necrosis occurs the cell membrane looses its integrity and becomes leaky (reviewed in Allen *et al.*, 1997). Therefore the measurement of Annexin V binding to the cell surface as indicative for apoptosis has to be performed in conjunction with a dye exclusion test such as 7-AAD to establish integrity of the cell membrane. Cells staining for Annexin V alone were considered in a stage of early apoptotic whereas cells double positive for Annexin V and 7-AAD were late apoptotic or necrotic (Vermes *et al.*, 1995).

Annexin V staining of cells was performed using an apoptosis detection kit following the manufacturer's instructions (Pharmingen). Briefly, freshly isolated cells or cells that had been in culture were washed twice in cold PBS (300 x g, 7min, 4°C), resuspended at 10^5 cells/100µl of the supplied 1x binding buffer, and added to flexible 96 round-bottom well plates (Becton Dickinson). 5µl of Annexin V-FITC or Annexin V-PE and 5µl 7-AAD were added per 10^5 cells and incubated in the dark at RT. Cells were washed once in 200µl of cold PBS and resuspended in 400µl 1x Binding Buffer. Cells were analysed with a Becton Dickinson FACSCalibur (BD Immunocytometry Systems).

II.3.10 Culturing of murine fibroblasts (L cells)

Untransfected L cells were grown in complete RPMI in tissue culture flasks (75 cm^2, Costar). I-A^{b+} L cells were grown in HAT+MX medium (Ronchese *et al.*, 1987). I-A^{b+} L cells co-transfected with Jagged1 or Delta1 were maintained in the selective medium HAT+MX + geneticin (section II.1). L cell lines were passaged by 1:5 subculture twice per week after

trypsin/EDTA treatment. All L cell transfectants were generously provided by K. Wong (Dept. Biology, Imperial College).

II.3.11 Trypsin/EDTA treatment of L cells

Media was completely removed from the tissue culture flasks prior to adding warm trypsin/EDTA (0.5µg/ml trypsin, 0.2µg/ml EDTA) for 5 min. For subculturing, detached cells were washed once with their respective medium. If used for experiments cells were washed three times with complete RPMI.

II.3.12 Mitomycin C treatment of L cells

L cells were pelleted by centrifuging at 300 x g for 7 min at RT and resuspended in 100µg/ml mitomycin C in complete RPMI at $2x10^6$ cells/ml. After incubation at 37°C for 1 hour 10 min, the cells were washed 3 times with complete RPMI.

II.3.13 Mixed lymphocyte reaction (MLR)

Bone marrow-derived DCs or mitomycin C-treated L cells were used as stimulator cells and plated out at differing cell numbers in a round bottom 96-well plate. T cells ($CD4^+$ and/or $CD8^+$ T cells) were added in complete RPMI at $2x10^5$/well to a total volume of 200µl. 96-well plates were incubated at 37°C in a humidified incubator with 5% CO_2 for 72 or 96 hrs and proliferation was measured by incorporation of 20µl/well of tritiated thymidine (1mCi/ml [6-^{3}H]Thymidine, Amersham Pharmacia) diluted 1:40 in complete RPMI 16 hrs before harvesting using a liquid scintillation counter Betaplate 1205 (Wallac). Each sample was tested in triplicate or more and plotted as cpm x 10^{-3} ± standard deviation.

For restimulation assays, flow cytometry and FACS, 0.5-1x10^6 mitomycin C-treated L cells and 3-4x10^6 T cells ($CD4^+$ and/or $CD8^+$ T cells) were added per 24-well in 2ml complete RPMI and incubated at 37°C in a humidified incubator with 5% CO_2 for 16 hrs to 5 days depending on the experiment.

II.3.14 Mitogen proliferation

Polyclonal mitogens used in this study were PMA with ionomycin and *E. coli* LPS. Briefly, single cell suspensions were obtained as previously described and plated out at 2x10^5 cells/well in 96-well plates or at 2x10^6 cells/well in 24-well plates. Mitogens were added to the wells at optimal concentrations, diluted in complete RPMI (PMA and ionomycin at 20ng/ml and 500ng/ml respectively, and LPS at 1μg/ml). Samples were incubated at 37°C in a humidified incubator with 5% CO_2 for 72 hrs and then harvested as described in section 1.3.13.

II.3.15 Polyclonal TCR stimulation using plate bound and soluble antibodies

Stimulation of purified T cell populations was performed using immobilised monoclonal antibodies against CD3ε and soluble monoclonal antibodies directed against CD28 (Pharmingen). Briefly, immobilisation of anti-CD3ε monoclonal antibodies onto plastic was performed by adding 30μl of the antibody at 1μg/ml in PBS per 96-well or 500μl of the antibody per 24-well. The plates were incubated for 16 hrs at 4°C or for 2 hrs at 37°C. Wells were washed 3 times with cold PBS to remove excess unbound antibody. Purified cells were plated out at 2x10^5 per 200μl/96-well or at 2x10^6 per 2ml/24-well in complete RPMI. Anti-CD28 monoclonal antibodies were added to the wells at a final concentration of 5μg/ml diluted in complete RPMI. Cells were incubated at 37°C in a humidified incubator with 5% CO_2 for 72 hrs and harvested as described.

II.4 Biochemical Methods

II.4.1 Enzyme linked immunosorbant assay (ELISA)

Cytokine secretion was analysed by detecting soluble protein in aliquots of supernatants collected from cultures at 24, 48 or 72 hrs using the DuoSet ELISA Development System (R&D Systems).

Briefly, anti-cytokine capture antibodies were diluted in PBS to the working dilution according to the manufacturer's instruction. 50µl of diluted capture antibody was added to the wells of enhanced protein binding ELISA plates (Corning). The plates were sealed and incubated overnight at RT. Capture antibody was removed and wells washed 3 times with ELISA wash buffer. 200µl of ELISA blocking buffer was added to each well and the plates incubated at RT for one hour. Wells were washed 3 times with ELISA wash buffer and 50µl of standards and samples were added to the wells (appropriately diluted in ELISA reagent diluent). Plates were sealed and incubated overnight at 4°C. Wells were washed 3 times with 200µl ELISA wash buffer and 50µl of detection antibody diluted in ELISA reagent diluent (manufacturer's instructions) was added to each well. Plates were incubated for 2 hrs at RT after which wells were washed 3 times with 200µl ELISA wash buffer. 50µl of Streptavidin-HRP diluted in ELISA reagent diluent (manufacturer's instructions) was added to the wells and incubated for 20 min at RT. Wells were washed 4 times with ELISA wash buffer and 50µl of TMB liquid substrate solution was added to wells. Plates were placed in the dark for colour change to occur and the reaction was stopped after 20 min by adding 25µl of 0.5M H_2SO_4. The plate was read on a MRX Reader (Dynatech Laboratories) and data analysed with Revelation Version 3.04 software. Data were expressed as pg/ml/10^6 cells.

III Expression of Notch components in T cells

III.1 Introduction

NF-κB exists in the cytoplasm of the majority of cell types and is important as a key regulatory molecule in the immune response (see section I.3), cell proliferation and cell survival (reviewed in Ghosh *et al.*, 1998). The DNA-binding form of NF-κB is dimeric and is composed of various combinations of five different DNA-binding subunits: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB and c-Rel. The most frequently observed form of NF-κB is the heterodimer p50/p65. In unstimulated cells, NF-κB is kept as an inactive form in the cytoplasm thorough interactions with a class of inhibitory proteins called IκBs. Recently Guan and Wang identified a region on the intracellular portion of Notch1 (NBD, Fig. I.8), which directly interacts with the p50 subunit of NF-κB and is responsible for ICN1-mediated inhibition of NF-κB transcriptional activity thereby displaying functional properties similar to IκB (Guan *et al.*, 1996; Wang *et al.*, 2001). In contrast, transgenic mice expressing ICN3 under the control of the Lck promoter display constitutive activity of NF-κB (Bellavia *et al.*, 2000). These mice developed aggressive T cell lymphomas with a NF-κB-dependent anti-apoptotic phenotype.

Furthermore, Notch can also induce expression of NF-κB-responsive genes. CBF1 normally represses expression of the NF-κB-dependent genes IL-6 and NF-κB2 in the absence of Notch signalling probably by competing with NF-κB for binding at overlapping sites (Cheng *et al.*, 2001; Oswald *et al.*, 1998; Palmieri *et al.*, 1999). Activated ICN1 converts CBF1 into a transcriptional activator and increases the expression of NF-κB2 (Oswald *et al.*, 1998). Notch receptors and ligands are also targets of NF-κB signalling. Using DNA microarrays, Notch1 was identified as a target of NF-κB signalling (Li *et al.*, 2001), whereas c-Rel expression in HeLa cells induced Jagged1 expression (Bash *et al.*, 1999).

Activation of T lymphocytes by triggering TCR and CD28 signalling or by stimulation with PMA/ionomycin results in the induction of NF-κB activity (see I.3 and Fig. I.4). Because of

the cross-regulation of NF-κB and Notch signalling, it is of interest to examine the expression of Notch pathway components in activated $CD4^+$ and $CD8^+$ T cells.

IL-10 controls inflammatory processes by suppressing the production of pro-inflammatory cytokines, which are known to be transcriptionally controlled by NF-κB. The molecular mechanisms involved in the IL-10 mediated inhibition of NF-κB still remain unclear. It is thought that IL-10 suppresses the activity of IκB kinases, which are required for the degradation of IκB and the subsequent nuclear translocation of NF-κB (Romano *et al.*, 1996; Wang *et al.*, 1995). Another mechanism emerges whereby IL-10 blocks the ability of translocated NF-κB to bind to DNA (Schottelius *et al.*, 1999). Therefore IL-10 may interfere with TCR/CD28 induced regulation of the expression of Notch pathway components.

III.2 Gene transcripts of Notch pathway components are present in $CD4^+$ and $CD8^+$ T cells

Several groups have detected Notch receptors, ligands and downstream components such as the Hes and Deltex genes in thymocytes (Bellavia *et al.*, 2000; Felli *et al.*, 1999; Hasserjian *et al.*, 1996; Kaneta *et al.*, 2000; Robey *et al.*, 1996). Less is known about their expression in peripheral $CD4^+$ and $CD8^+$ T cells. It was, therefore, of interest to investigate the expression of the Notch pathway components in these T cell subsets isolated from peripheral lymphoid organs.

$CD4^+$ and $CD8^+$ T cells were purified by MACS from spleens of BALB/c mice. Total RNA was extracted and gene expression of Notch receptors and ligands were analysed by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. III.1A). For most genes analysed, there was very little difference in expression of Notch receptors and ligands between $CD4^+$ and $CD8^+$ T cells. Both expressed Jagged1, Jagged2, Notch1 and Hes1 at an intermediate and Notch2 at a high level. Differential expression was only found for Delta1 and Notch4: Delta1

transcripts were more abundant in $CD4^+$ T cells, whereas Notch4 was slightly more expressed in $CD8^+$ T cells.

Semi-quantification by conventional RT-PCR has a number of technical difficulties. As the PCR greatly amplifies the target, errors are also amplified. As a result, variability can be very large. Further, semi-quantification has to be made using values of the exponential phase of the reaction where no factor is limiting and the amplification products accumulate at a steady rate. This is difficult to achieve by RT-PCR. Therefore, I made use of real-time PCR (described in materials & methods, section II.2.21), which detects the amplification products while they are formed.

Total RNA of $CD4^+$ and $CD8^+$ T cells isolated from BALB/c spleens was transcribed into cDNA and analysed by real-time PCR (Fig. III.1B). As found by RT-PCR, most of the Notch components are present and many did not differ greatly between $CD4^+$ and $CD8^+$ T lymphocytes. Again Delta1 expression was consistently higher in $CD4^+$ T cells. Notch4 transcripts were 3-fold increased in $CD8^+$ T cells compared to $CD4^+$ T cells.

Notch receptor and ligands, as well as downstream components are present in peripheral $CD4^+$ and $CD8^+$ T cells. This raises the question as to whether the Notch pathway is important not only for T cell development but also in the regulation of immunity in the adult perhaps by influencing T cell activation, polarisation and/or apoptosis.

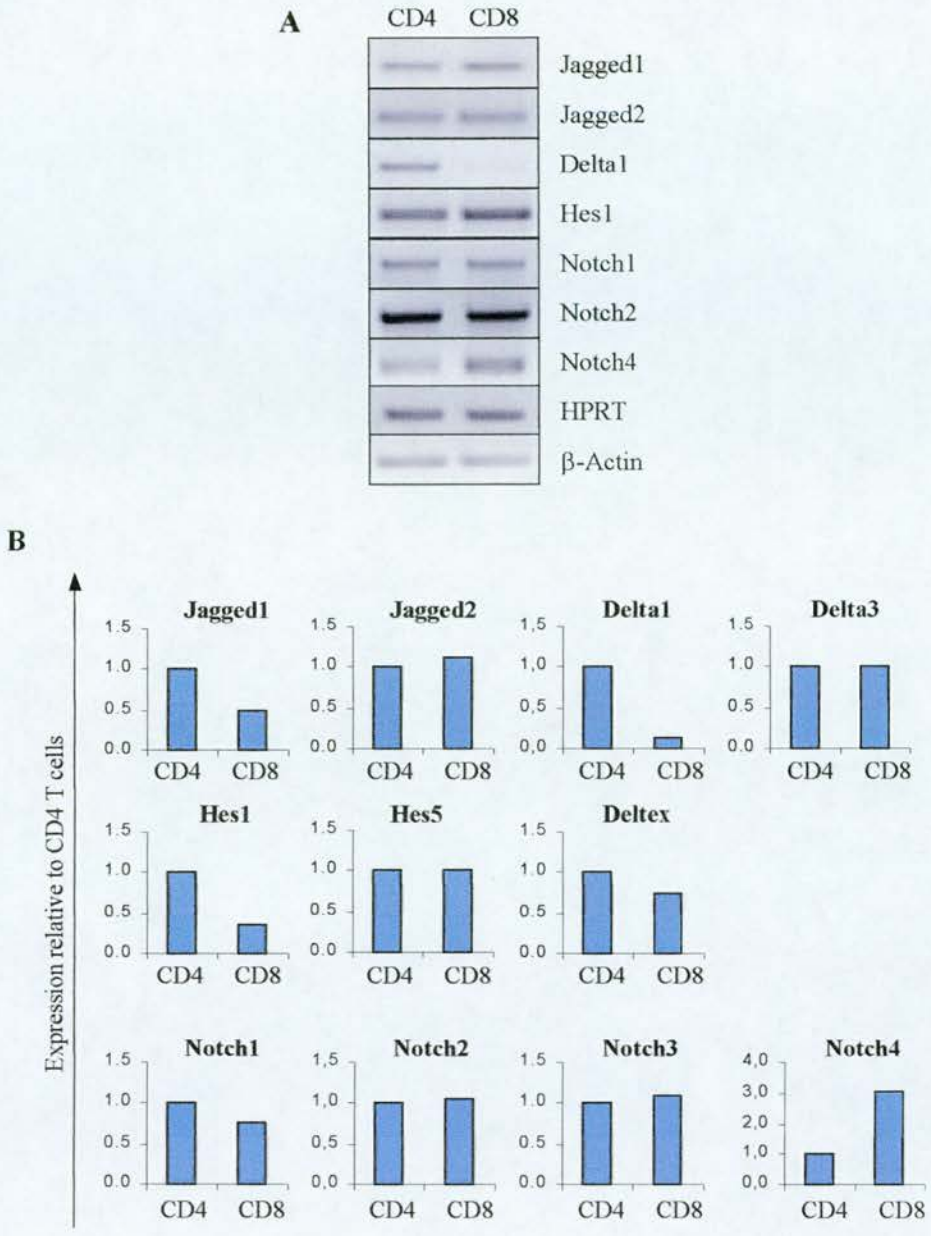


Figure III.1. Gene transcripts of Notch pathway components are present in $CD4^+$ and $CD8^+$ T cells.

$CD4^+$ and $CD8^+$ T cells were MACS purified from BALB/c spleens. Total RNA was extracted and quality was tested by RT-PCR as described in materials & methods, section II.2.16.

[A] The RNA was reverse transcribed and amplified by RT-PCR. The PCR-amplified products were run in a 2% agarose gel. β-Actin was used as a house keeping gene to normalise all the RNA samples to the same amount of starting template and as a control for the PCR reaction itself.

[B] RNA was reverse transcribed to cDNA and amplified by real-time PCR as described in materials & methods, section II.2.21. Levels of mRNA transcripts are shown relative to the levels of the internal calibrator which were $CD4^+$ T cells. A change in expression of at least 3-fold or greater was considered significant.

III.3 Activation of $CD4^+$ T cells leads to differential gene expression of Notch pathway components

It has been reported that the activated form of Notch1 is a specific inhibitor of NF-κB complexes containing the p50 subunit (Guan *et al.*, 1996; Wang *et al.*, 2001). Thus, Notch signalling through its effect on NF-κB could play a role in regulating an immune response. Furthermore, c-Rel and RelA, both members of the NF-κB family, induced Jagged1 transcription in a HeLa-derived cell line (Bash *et al.*, 1999). To confirm this data, the authors stimulated human Jurkat T cells with phorbol 12-myristate 13-acetate (PMA) plus ionomycin to activate endogenous NF-κB. This treatment also led to an increase in Jagged1 message.

Naturally occurring $CD4^+CD25^+$ regulatory T cells express high levels of Deltex1 and upregulate gene expression of Delta1, Notch4 and Hes1 upon *in vitro* stimulation with anti-CD3ε/anti-CD28, whereas Deltex1 was downregulated (Ng *et al.*, 2001). These cells resemble anergic cells *in vitro* and can suppress the responses of $CD4^+CD25^-$ T cells in coculture in a cell contact-dependent manner (Dieckmann *et al.*, 2001; Thornton and Shevach, 1998). The induction of anergic T cells has been demonstrated *in vitro* (Groux *et al.*, 1996; Jenkins and Schwartz, 1987; Lamb *et al.*, 1983; Lombardi *et al.*, 1994). Some of the protocols to induce anergic or hyporesponsive T cells involve the presence of IL-10 (Groux *et al.*, 1996; Groux *et al.*, 1997).

***In vitro* activation of $CD4^+$ T cells.**

To investigate the effect of activation on expression of Notch components, $CD4^+$ T cells were MACS-purified from BALB/c spleens and activated by either plate-bound anti-CD3ε antibodies in the presence of soluble anti-CD28 antibodies or by treatment with PMA and ionomycin (Fig. III.2). IL-10 was included in the stimulation with anti-CD3ε/anti-CD28 to examine whether expression of the Notch genes is affected by a regulatory/immunosuppressive cytokine. Proliferation was measured after three days of activation. Both, anti-CD3ε/anti-CD28 and PMA/ionomycin stimulation induced $CD4^+$ T cell proliferation (Fig. III.2A), but at different magnitudes. The presence of IL-10 did not

influence proliferation in this experiment, whereas in other experiments a small decrease in proliferation was observed. However, IL-10 downregulated production of IL-2 and IFNγ but not IL-4 as measured by ELISA (Fig. III.2B-D). Even though PMA/ionomycin induced strong proliferation and IL-2 production, IFNγ and IL-4 levels were two- and six-fold, respectively, lower compared to the anti-CD3ε/anti-CD28 treatment.

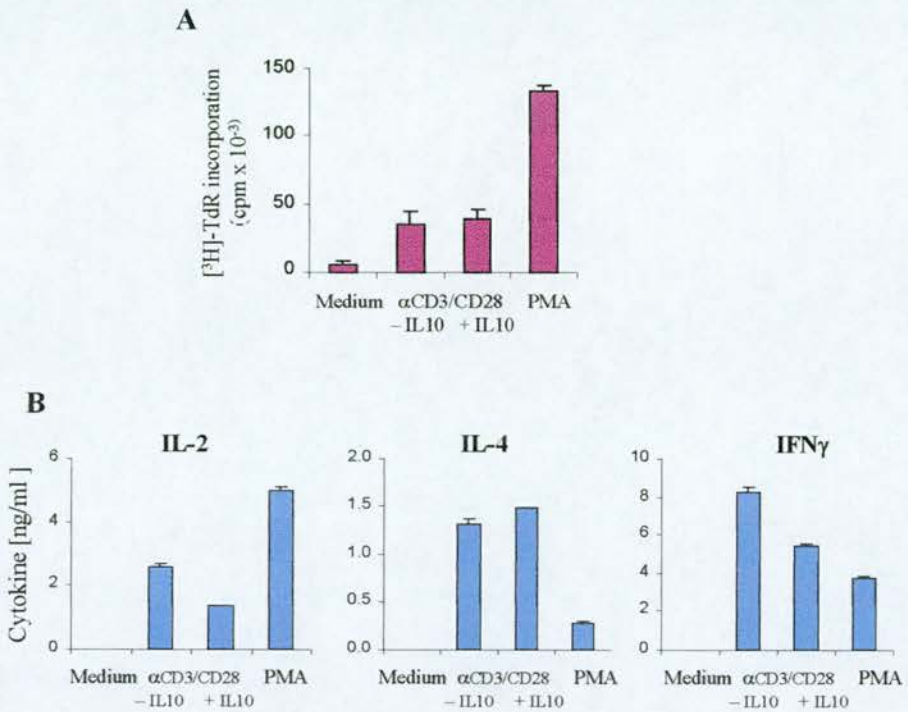


Figure III.2. *In vitro* activation of CD4$^+$ T cells.
CD4$^+$ T cells were MACS purified from BALB/c spleens and activated with 1μg/ml anti-CD3 and 5μg/ml anti-CD28 in the presence or absence of 50ng/ml IL-10 (+/– IL-10) or with 50ng/ml PMA and 500ng/ml ionomycin.
[A] After 3 days, proliferation was measured by [^{3}H]thymidine uptake during the last 8 hrs of culture and plotted as cpm x 10^{-3} ± standard deviation.
[B] Culture supernatants were harvested after 24 hrs (IL-2) and after 48 hrs (IL-4 and IFNγ) to measure cytokine contents by ELISA.

Activating $CD4^+$ T cells *in vitro* leads to differential gene expression of Notch pathway components and upregulation of cytokine transcripts.

Total RNA was extracted from $CD4^+$ T cells *ex vivo* (0 hrs) or after 24 or 48 hours of their respective stimulation and gene expression was analysed by real-time PCR (Fig. III.3). The cytokine pattern observed for IFNγ and IL-4 matched the results obtained by ELISA. However, whereas considerable amounts of IL-2 were detected in the supernatant of cells stimulated with PMA/ionomycin, only a little was detected at the RNA level compared to anti-CD3ε/anti-CD28 activated cells. The decrease of IL-2 induced by IL-10 was also observed by real-time PCR analysis. Jagged1 and Delta1 transcripts were downregulated, whereas Jagged2 transcripts were induced after 48 hours of any stimulation. Decrease in Delta3 expression did not reach significance except at the 24-hour time point of PMA/ionomycin activation. The Notch receptors 1 and 2 did not change greatly their expression upon any stimulation. All activated $CD4^+$ T cells showed a pronounced downregulation of Hes1 and Deltex1, whereas Hes5 was strongly upregulated at 24 hours upon stimulation in the presence of IL-10.

Stimulation of $CD4^+$ T cells with anti-CD3ε/anti-CD28 or PMA/ionomycin induced proliferation, cytokine production and a pronounced downregulation of Delta1, Deltex1 and the downstream target of Notch signalling Hes1. The presence of IL-10 during stimulation affected cytokine production but not proliferation. Furthermore, most of the Notch components did not alter their expression in the presence of IL-10 compared to activation in the absence of IL-10. Hes5 was the only gene analysed which showed a transient increase in the presence of IL-10.

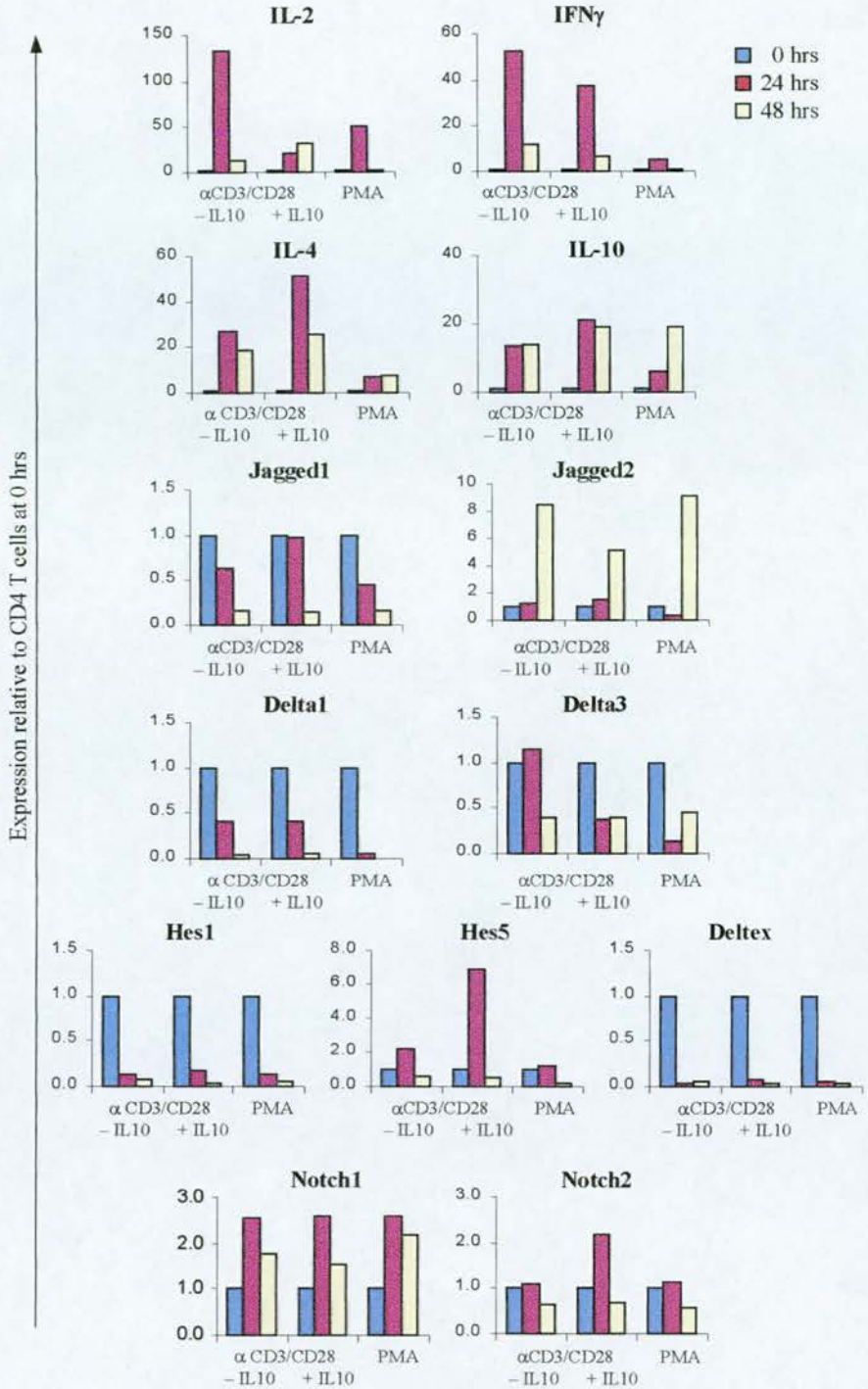


Figure III.3. Activating CD4$^+$ T cells *in vitro* leads to differential gene expression of Notch pathway components and upregulation of cytokine transcripts.
CD4$^+$ T cells were MACS purified from BALB/c spleens and activated with 1μg/ml anti-CD3 and 5μg/ml anti-CD28 in the presence or absence of 50ng/ml IL-10 (αCD3/CD28 +/– IL-10) or with 50ng/ml PMA and 500ng/ml ionomycin (PMA). Cells were collected directly after purification (0 hrs) and 24 or 48 hrs after activation and lympholyte-treated. Total RNA was extracted and quality was tested by RT-PCR as described in materials & methods, section II.2.16. RNA was reverse transcribed to cDNA and analysed by real-time PCR. Levels of mRNA transcripts are shown relative to the levels of the internal calibrator which were CD4$^+$ T cells taken at 0 hrs. A change in expression of at least 3-fold or greater was considered significant.

Purification by positive selection using MACS-beads does not influence gene expression of Notch pathway components in $CD4^+$ T cells.

Generally, many genes of the Notch pathway were downregulated with all treatments compared to $CD4^+$ T cells *ex vivo*. This raises concerns whether culturing of the cells alone is responsible for this finding. Additionally, positive selection of $CD4^+$ T cells by MACS purification may partially activate them. Therefore, an additional experiment was carried out where two different purification methods were compared.

$CD4^+$ T lymphocytes were isolated from BALB/c spleens either by positive selection using MACS microbeads or negative selection (depletion) using R&D columns. Cells were activated with PMA/ionomycin and proliferation measured after 3 days (Fig. III.4A). The magnitude of proliferation did not differ between the two purification protocols. However, positive selection induced a small background proliferation in $CD4^+$ T cells incubated in medium. Gene expression of *ex vivo* $CD4^+$ T lymphocytes, cells incubated in medium or treated with PMA/ionomycin for 24 hours were analysed (Fig. III.4B). The expression pattern for Jagged1, Deltex1, Hes1 and Hes5 were very similar between $CD4^+$ T cells purified by positive or by negative selection and only a maximal twofold difference in scale was observed between the two purification protocols. This suggests that positive selection did not affect gene expression of Notch pathway components. Culturing of $CD4^+$ T cells strongly decreased expression of Deltex1 and Hes1. PMA/ionomycin stimulation increased Hes1 transcripts but did not influence Deltex1 expression compared to cells cultured in medium. Expression of Jagged1 did not decrease significantly upon culturing in media but was further downregulated after activation. Hes5 did not alter expression upon culturing or stimulation.

Positive selection of $CD4^+$ T cells did not affect gene expression of Notch pathway components. In contrast, culturing the cells was responsible for the downregulation seen for Deltex1 and Hes1. Taking experiment III.4 into account, activation of $CD4^+$ T cells seemed to induce Hes1 compared to cells resting in medium or to inhibit downregulation of Hes1 compared to *ex vivo* $CD4^+$ T cells. In summary, I have shown that certain genes of the Notch pathway were differentially regulated upon activation implying a role for Notch signalling in $CD4^+$ T cells during induction of an immune response.

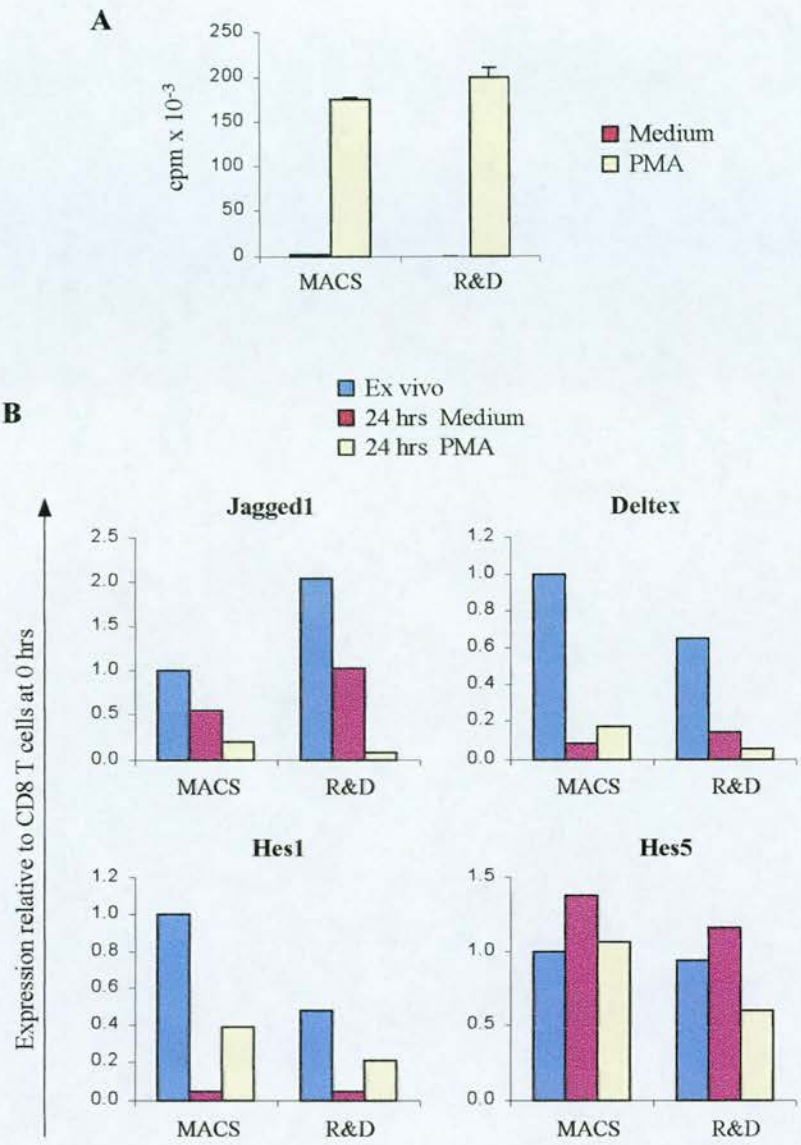


Figure III.4. Purification by positive selection using MACS-beads does not influence gene expression of Notch pathway components in $CD4^+$ T cells.

$CD4^+$ T cells were purified from BALB/c spleens by positive selection using MACS or negative selection using the R&D kit. $CD4^+$ T cells were cultured in medium (Medium) or activated with 50ng/ml PMA and 500ng/ml ionomycin (PMA).

[A] After 3 days, proliferation was measured by [^{3}H]thymidine uptake during the last 8 hrs of culture and plotted as cpm x 10^{-3}.

[B] Cells were collected directly after purification (Ex vivo) or after 72 hrs of culturing (72 hrs Medium & 72 hrs PMA) and lympholyte-treated. Total RNA was extracted and quality was tested by RT-PCR as described in materials & methods, section II.2.16. RNA was reverse transcribed to cDNA and analysed by real-time PCR. Levels of mRNA transcripts are shown relative to the levels of the internal calibrator which were MACS purified $CD4^+$ T cells taken at 0 hrs (Ex vivo). A change in expression of at least 3-fold or greater was considered significant.

III.4 Activation of CD8$^+$ T cells leads to differential gene expression of Notch pathway components

I have shown that components of the Notch pathway were differentially transcribed in CD4$^+$ T cells upon culturing and activation. The presence of these genes in CD8$^+$ T cells (Fig. III.1) suggests that they may also play a role during an activation process.

***In vitro* activation of CD8$^+$ T cells.**

CD8$^+$ T cells were MACS-purified from BALB/c spleens and activated by either plate-bound anti-CD3ε and soluble anti-CD28 antibodies or by treatment with PMA and ionomycin (Fig. III.5). Proliferation was measured after three days of activation. CD8$^+$ T lymphocytes proliferated strongly to both stimuli. However, a small background proliferation of cells incubated in medium alone was observed. This may be due to the positive selection by the anti-CD8 MACS microbeads.

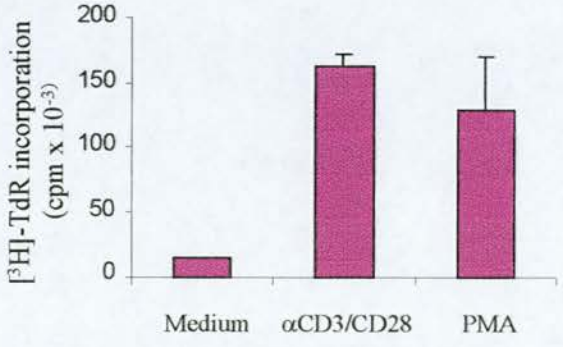


Figure III.5. *In vitro* activation of CD8$^+$ T cells.
CD8$^+$ T cells were MACS purified from BALB/c spleens and activated with 5μg/ml anti-CD3 and 5μg/ml anti-CD28 or with 50ng/ml PMA and 500ng/ml ionomycin. After 3 days, proliferation was measured by [^{3}H]thymidine uptake during the last 8 hrs of culture and plotted as cpm x 10^{-3} ± standard deviation.

Activating $CD8^+$ T cells *in vitro* leads to differential gene expression of Notch pathway components and upregulation of cytokine transcripts.

After 0 (*ex vivo*), 24 and 48 hours of stimulation total RNA was extracted and gene expression was analysed by real-time PCR (Fig. III.6). Jagged1 and Delta1 were downregulated, whereas Jagged2 was upregulated after 48 hours of PMA/ionomycin stimulation of the $CD8^+$ T cells as it was observed for $CD4^+$ T lymphocytes. Differential expression of Delta3, Notch1 and Notch2 did not exceed a three-fold change. Expression patterns of Hes1 and Deltex1 were almost identical between $CD4^+$ and $CD8^+$ T lymphocytes, in both cell types being strongly downregulated upon stimulation. Hes5 was the only gene exhibiting a different regulation of transcription between $CD4^+$ and $CD8^+$ T cells: in contrast to $CD4^+$ T lymphocytes, $CD8^+$ T cells upregulated Hes5 transcripts after 48 hours of PMA/ionomycin treatment.

Similar to $CD4^+$ T cells, $CD8^+$ T lymphocytes differentially regulate expression of Notch pathway components. They also have strongly decreased expression of Hes1 and Deltex1 transcripts after being activated. However, I cannot exclude that the culturing itself rather than the activation was responsible for the observed downregulation.

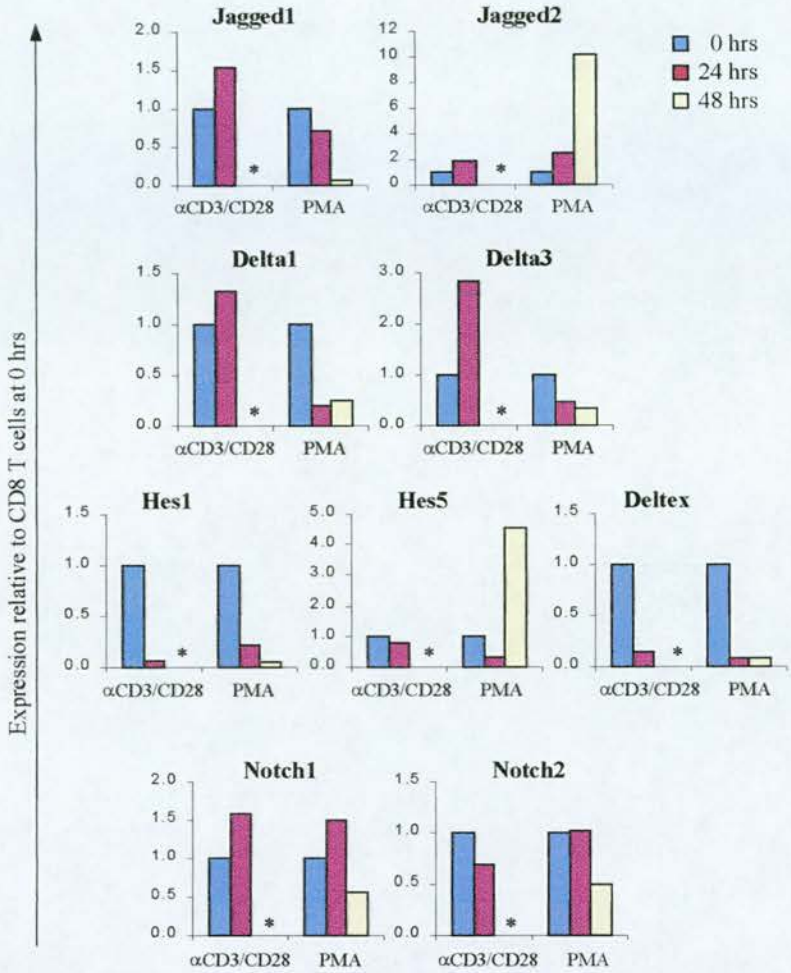


Figure III.6. Activating CD8$^+$ T cells *in vitro* leads to differential gene expression of Notch pathway components and upregulation of cytokine transcripts.

CD8$^+$ T cells were MACS purified from BALB/c spleens and activated with 5μg/ml anti-CD3 and 5μg/ml anti-CD28 (αCD3/CD28) or with 50ng/ml PMA and 500ng/ml ionomycin (PMA). Cells were collected directly after purification (0 hrs) and 24 or 48 hrs after activation and lympholyte-treated. Total RNA was extracted and quality was tested by RT-PCR as described in materials & methods, section II.2.16. RNA was reverse transcribed to cDNA and analysed by real-time PCR. Levels of mRNA transcripts are shown relative to the levels of the internal calibrator which were CD8$^+$ T cells taken at 0 hrs.

*) Sample for 48 hrs of αCD3/CD28 activation was not determined. A change in expression of at least 3-fold or greater was considered significant.

III.5 Discussion

Components of the Notch pathway have been studied extensively during T cell development (Deftos *et al.*, 2000; Radtke *et al.*, 1999; Robey *et al.*, 1996). Less is known about their expression in peripheral T lymphocytes. Here, I have investigated the expression of Notch receptors, ligands and downstream components in peripheral $CD4^+$ and $CD8^+$ T cells. Notch receptors, ligands and targets of Notch signals were all expressed in resting T cells. *In vitro* culturing with anti-CD3/anti-CD28 antibodies, PMA/ionomycin or in medium alone reduced expression levels of Hes1 and Deltex1, both targets of Notch activation, implying abrogated Notch signalling. Therefore, I suggest that accessory cells are required to induce and/or sustain Notch signalling in peripheral T lymphocytes.

Naïve peripheral $CD4^+$ and $CD8^+$ T cells circulate through the blood and peripheral lymphoid organs in a proliferatively and transcriptionally quiescent state until they encounter an APC bearing a peptide bound to an appropriate MHC molecule. Activation of the TCR/CD3 complex by its natural ligand (MHC/peptide) or by crosslinking with anti-CD3 antibodies leads to rapid activation of NF-κB. NF-κB proteins are retained in the cytoplasm of unstimulated cells in an inactive form via interactions with one or more of the seven known IκB proteins (reviewed in Baldwin, 1996). In response to a variety of stimuli, including cell-surface antigen receptor cross-linking and exposure to cytokines, IκB proteins are phosphorylated and degraded and free cytoplasmic NF-κB dimers translocate rapidly to the nucleus, where they regulate κB-dependent gene expression (May and Ghosh, 1998). Notch1 has been reported to inhibit NF-κB-induced gene expression similarly to IκB proteins (Guan *et al.*, 1996) by binding to the NF-κB p50 subunit and blocking its DNA binding activity (Wang *et al.*, 2001). Interestingly, NF-κB activates Notch signalling in neighbouring cells by inducing expression of Jagged1 on the cell surface (Bash *et al.*, 1999). This demonstrates that the cross-talk between Notch and NF-κB is bi-directional, meaning that the activation of one can influence the activation of the other.

Others and I have shown that Notch receptor, ligands and downstream components are present in peripheral $CD4^+$ and $CD8^+$ T cells (Fig. III.1; Hoyne *et al.*, 2000; Ng *et al.*, 2001). Activation of T cells by crosslinking CD3 or stimulation with PMA/ionomycin activates NF-

κB (Kuo and Leiden, 1999). In contrast to the finding of Bash *et al.*, Jagged1 expression was downregulated upon stimulation in both $CD4^+$ and $CD8^+$ T cells (Fig. III.3 & 6). There are several possible reasons for the contradicting results. It has been shown that Notch signalling has different outcomes depending on the cell type: Notch activation protects T cells from TCR-mediated apoptosis (Jehn *et al.*, 1999), but induces apoptosis in B cells (Morimura *et al.*, 2000). Bash and colleagues used HeLa cells, which were derived from a human epithelial carcinoma, to induce Jagged1 expression by transfecting them with NF-κB (Bash *et al.*, 1999). However, they also detected Jagged1 expression in Jurkat cells after two hours of stimulation with PMA/ionomycin. In my system, the decrease in Jagged1 was detected at 24 hours or later after stimulation, which may be the reason for the discrepancy.

The strong downregulation of Hes1 and Deltex1 upon culturing of T cells with or without stimulation (Fig. III.3 & 4) suggests the absence of Notch signalling. *In vivo* T lymphocytes are in contact with many cells and cell types, which may allow Notch activation. Upon culturing in flat bottom wells, most of the T cells were not in contact with other cells, which may explain the decreased level of targets of the Notch signalling pathway. Changes of Hes5 expression in the case of $CD4^+$ T cells activated in the presence of IL-10 would then be caused by the signalling cascade from the TCR/CD3 complex, CD28 costimulation and/or signalling by IL-10 rather than by Notch ligands expressed on adjacent cells. However, cell-to-cell contact cannot be excluded as the reason for Hes5 upregulation.

The details of IL-10 signalling remain to be resolved. Distinct signalling pathways have been described, one involving the JAK/STAT system and another one leading to modulation of NF-κB activity (Moore *et al.*, 2001). In $CD4^+$ T cells and macrophages, IL-10 inhibits NF-κB activation (Romano *et al.*, 1996; Wang *et al.*, 1995), whereas IL-10 activates AP-1 and NF-κB in $CD8^+$ T cells (Hurme *et al.*, 1994). This is consistent with the finding that IL-10 has no direct inhibitory effects on the proliferation of $CD8^+$ T cells activated by anti-CD3 antibodies (Groux *et al.*, 1998). Therefore, and because IL-10 mediated anergy can be associated with induction of a population of regulatory $CD4^+$ T lymphocytes (de Waal Malefyt *et al.*, 1993; Groux *et al.*, 1996; Groux *et al.*, 1997), the effect of IL-10 was investigated for $CD4^+$ T cells only. Even though IL-10 did not inhibit the proliferative response of $CD4^+$ T cells to anti-CD3 antibody-mediated stimulation, it downregulated production of IL-2 transcripts and protein (Fig. III.2 & 3) consistent with previous reports (de Waal Malefyt *et al.*, 1993; Taga *et al.*,

1993). The differences in IL-2 transcripts between anti-CD3/anti-CD28 activated and PMA/ionomycin activated $CD4^+$ T cells may be explained by different kinetics. Phorbol esters such as PMA directly activate protein kinase C (PKC) whereas the calcium ionophore ionomycin increases cytosolic free Ca^{2+}. In combination, they fully activate transcription factor NF-AT, which is required for IL-2 gene transcription (reviewed in Jain *et al.*, 1995). These are downstream events of TCR signalling, which may be more rapidly induced by pharmacological agents than by anti-CD3/anti-CD28 antibody stimulation. Therefore, IL-2 transcripts may have already decreased 24 hours after stimulation with PMA/ionomycin.

In summary, it can be concluded that Notch receptors and ligands are expressed in $CD4^+$ and $CD8^+$ T cells. Activation of T lymphocytes did not have a great effect on Notch1 and 2. However, the ligands and downstream components were differentially regulated upon culturing and/or stimulation. The marked decrease of Hes1 and Deltex1 transcripts in $CD4^+$ T cells upon culturing for 24 hours in medium suggests abrogated Notch signalling. Activation of $CD4^+$ T cells only partially restored Hes1 transcription. The presence of accessory cells expressing the Notch ligands may be required for sustained expression of Hes1 and/or Deltex1. The capacity of IL-10 to increase Hes5 transcription suggests that cytokines additionally to Notch ligands may modulate gene expression of Notch target genes. For certain cytokines a direct involvement in Notch signalling has been shown: G-CSF and GM-CSF modulate signalling by Notch1 and Notch2, respectively (Bigas *et al.*, 1998).

The pronounced differential expression of Hes1 and Deltex1, both targets of Notch activation (Deftos *et al.*, 1998; Deftos *et al.*, 2000; Jarriault *et al.*, 1995; Jarriault *et al.*, 1998), suggests a role for T lymphocytes as the receiving cells of Notch signalling. This poses the question of the identity of the signalling cell expressing the Notch ligands. In order to become activated, T cells require an encounter with antigen-presenting cells (APCs), which may activate Notch signalling in T cells during the activation process. To test this hypothesis, expression of Notch pathway components in dendritic cells (DCs), since they are the most potent APCs, were examined.

IV Expression of Notch components in dendritic cells

IV.1 Introduction

Notch receptors play a pivotal role in survival, expansion and differentiation of haematopoietic precursors. Less is known about their role in differentiated DCs. Jagged1 expressed on APCs inhibited an immune response *in vivo* (Hoyne *et al.*, 2000) implying a role for Notch signalling in the induction of tolerance. I and other investigators have shown that Notch receptors are present in $CD4^+$ and $CD8^+$ T lymphocytes (Chapter III; Hoyne *et al.*, 2000; Ng *et al.*, 2001) thereby enabling these cells to receive Notch signalling. DCs are an ideal candidate for delivering Notch signalling to T cells due to their central role in the induction and regulation of immune responses. Therefore, Notch expression was investigated in DCs, which were matured under various conditions. Whereas downstream components of the Notch pathway were hardly detectable, expression of the Notch ligands Jagged1 and Delta1 was differentially regulated. Thus, there may be a potential role for Notch ligands in the interaction of DCs with other cell types, for example T lymphocytes.

DCs play a critical role in the initiation of many adaptive immune responses. They exist in a quiescent state in most tissues, but certain stimuli such as microbial products or inflammatory cytokines trigger DCs to become activated. They then migrate to the draining lymph nodes, where they encounter T lymphocytes and induce an immune response.

DCs develop in the bone marrow (BM) and then migrate to the periphery (Barclay and Mayrhofer, 1981; Katz *et al.*, 1979). Members of the Notch family are expressed in lymphoid, myeloid and erythroid precursors of the BM and play crucial roles during haematopoiesis (Milner and Bigas, 1999). The first evidence for regulation of Notch signalling by cytokines came from studies of myeloid differentiation (Bigas *et al.*, 1998): Differentiation of myeloid precursors was inhibited by Notch1 in response to G-CSF or by Notch2 in response to GM-CSF. This led to identification of the Notch cytokine response (NCR) region on the cytoplasmic portion of Notch. Furthermore, an immobilised truncated form of Delta1 induced apoptosis in peripheral blood monocytes cultured with M-CSF, but not GM-CSF (Ohishi *et*

al., 2000). These observations highlight a potentially important link between Notch and cytokine signalling pathways.

However, members of the Notch family are not only important in the haematopoiesis of DCs, but also in instructing T cell responses. Splenic DCs express Notch1 and Jagged1 (Hoyne *et al.*, 2000). Overexpressing Jagged1 in a DC-enriched APC population may induce peripheral $CD4^+$ T lymphocytes to become regulatory cells (Hoyne *et al.*, 2000). There is increasing evidence that certain subpopulations of DCs, such as liver-derived DCs (Thomson and Lu, 1999), $CD8\alpha^+$ lymphoid-derived DCs (Kronin *et al.*, 2000) and IL-10 modulated DCs (Steinbrink *et al.*, 1999; Steinbrink *et al.*, 1997) are able to downregulate immune responses. It is, therefore, relevant to investigate the regulation of expression of Notch pathway components in DCs.

DCs comprise as few as 0.5% of the nucleated cells in nonlymphoid tissues while lymphoid tissues contain slightly elevated numbers (around 1%) (Vremec *et al.*, 1992). Time-consuming purification procedures involving density gradient centrifugation, adherence to glass or plastic surfaces, panning, depletion or positive selection using magnetic beads and cell sorting have been developed to obtain significant quantities of tissue-derived DCs. Purity and overall yield are usually quite low. *In vitro* differentiation of DCs from murine bone marrow following Scheicher's or Inaba's method generates a large quantity of highly enriched DCs (Inaba *et al.*, 1992a; Scheicher *et al.*, 1992). Therefore, these two methods of BM-derived DC cultures were employed to study the gene expression of Notch family members.

IV.2 Differentiation of bone marrow-derived DCs based on Scheicher's protocol

While B lymphocytes and pre-B cells do not grow in response to GM-CSF, they represent about 50% of the initial marrow suspension (Inaba *et al.*, 1992a). Mainly three cell types of myeloid cells expand in mouse bone marrow cultures in the presence of GM-CSF: (a) Neutrophils predominate but they do not adhere to the culture surface. (b) Macrophages are firmly adherent and express substantial levels of F4/80 antigen and little or no MHC class II. (c) DCs are released from cellular aggregates that are attached to the marrow stroma (Inaba *et al.*, 1992a). The protocol used for the following study was based on the method described by

Scheicher *et al.* (Scheicher *et al.*, 1992) and further optimised by using some of the procedures introduced by Lutz *et al.* (Lutz *et al.*, 1999): The overall culturing time was increased, whereas the amount of GM-CSF was decreased from day 6 onwards favouring the generation and survival of DCs over granulocytes and B cells.

The most common stimuli used to activate DCs are TNFα and LPS. Moreover TNFα has been widely applied to generate *in vitro* bone marrow-derived DCs. It was, therefore, used to mature the immature DC (iDCs) in combination with the regulatory cytokine IL-10. Steinbrink and colleagues reported that treatment of DCs with 40 to 80ng/ml IL-10 achieved maximal inhibition of allogeneic MLRs (Steinbrink *et al.*, 1997). Therefore, 50ng/ml IL-10 was used throughout the study.

IV.2.1 Bone marrow-derived DCs were matured in the presence or absence of IL-10

Bone marrow cells from C67BL/6 mice were cultured in GM-CSF as described in materials & methods, section II.3.1.2. At day 10 of culture, an aliquot of the non-adherent immature DCs (iDCs) was kept for analysis by flow cytometry and the rest was transferred into a 24-well plate for maturation with TNFα in the presence or absence of IL-10 (mature DCs ± IL-10). After 64 hours, matured DCs were analysed by flow cytometry (Fig. IV.1). After 10 days the cultured DCs (iDCs) still had a relatively immature phenotype: The majority of the cells expressed no or low levels of the costimulatory molecule B7.2 and intermediate levels of MHC class II molecules. TNFα-induced maturation increased the number of CD11c-positive cells from 78% to 94%. The population of cells expressing intermediate level of class II and low levels of B7.1 disappeared almost completely. Instead over 90% of the cells had B7.2^{high} class II^{high} phenotype.

There was a slight reduction in the levels of activation markers in DCs that were matured in the presence of TNFα and IL-10.

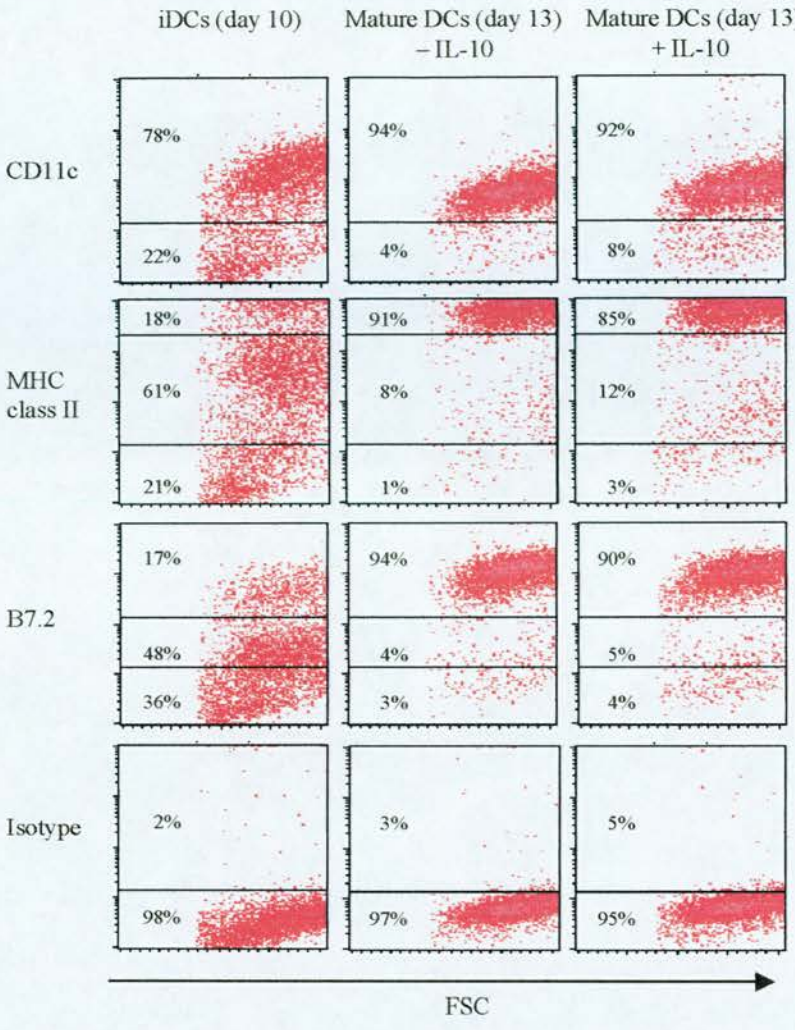


Figure IV.1. Bone marrow-derived DCs were matured in the presence or absence of IL-10 and analysed by flow cytometry.

Bone marrow-derived DCs from C57BL/6 mice were grown for 10 days and then matured with 50ng/ml TNFα for 64 hrs in the presence or absence of 50ng/ml IL-10 (Scheicher *et al.*, 1992). Immature DCs (day 10) and DCs matured in the presence (day 13, + IL-10) or absence IL-10 (day 13, – IL-10) were stained for CD11c and activation markers B7.2 and MHC class II and analysed by flow cytometry. Regions were set using isotype staining and to differentiate between low and high expressing cell populations for MHC class II and B7.2 staining. Numbers in the plots represent percentages of cells within the region. Details of antibodies are given in table II.4 & II.5.

IV.2.2 DCs matured in the presence IL-10 are less potent stimulators of allogeneic T cell proliferation

An exquisite function of DCs is their ability to stimulate naïve allogeneic T lymphocytes as evidenced by a mixed lymphocyte reaction (MLR) (Nussenzweig and Steinman, 1980). To examine the stimulatory capacity of DCs matured in the presence or absence of IL-10, C56BL/6 bone marrow cells were cultured for 10 days and then matured for 64 hours as described above. The matured DCs were cocultured with alloreactive T cells enriched from BALB/c spleens by depletion of granulocytes, B cells and class II-positive cells using Dynabeads. Control T cells and DCs were incubated in medium. Figure IV.2 represents the proliferative response of the T cells after 4 days of coculturing with DCs. As few as 100 TNFα-matured BM-derived DCs were sufficient to induce a marked proliferative response in T cells during a MLR. A significant inhibitory effect on T cell proliferation induced by DCs matured in the presence of IL-10 was detected at DC concentration 10^3 and 10^4 per well ($P<0.01$ and $P<0.05$, respectively).

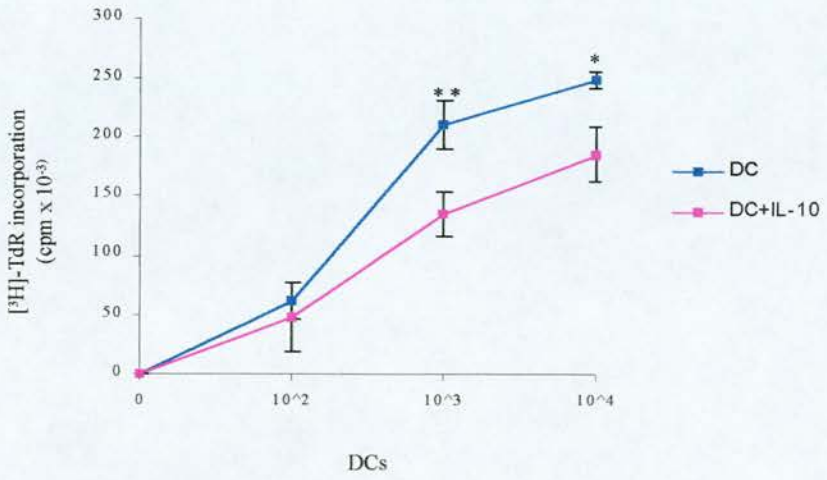


Figure IV.2. DCs matured in the presence IL-10 are less potent stimulators of allogeneic T cell proliferation than those matured in the absence of IL-10.
Bone marrow-derived DCs from C57BL/6 mice were grown and matured for 64 hrs as described in figure legend IV.1. A MLR was carried out with varying numbers of DCs as stimulators and depleted T cells from BALB/c spleens (10^5/well). After 4 days, proliferation was measured by [^{3}H]thymidine uptake during the last 18 hrs of culture and plotted as cpm x 10^{-3} ± standard deviation.
* : $P<0.05$; ** : $P<0.01$, statistical comparison of sample data was performed by application of ANOVA Test using the GraphPad InStat programme (GraphPad Software Inc., www.graphpad.com)
DC = DCs matured in the absence of IL-10; DC + IL-10 = DCs matured in the presence IL-10.

IV.2.3 Maturation of DCs with TNFα in the presence or absence of IL-10 leads to differential gene expression of Notch pathway components

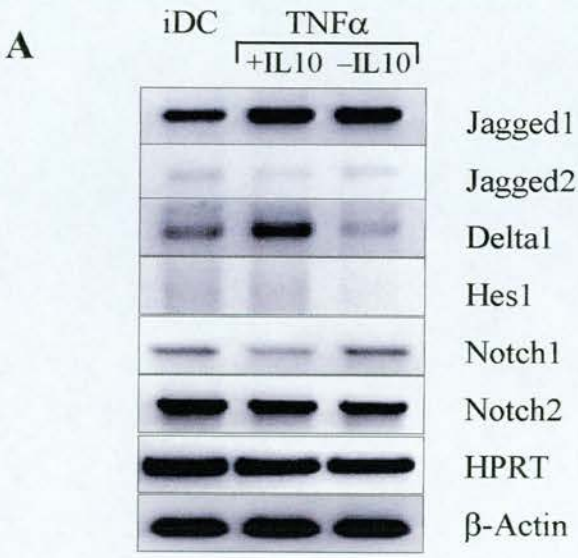
C56BL/6 bone marrow cells were cultured for 10 days and then matured for 64 hours as described above. Total RNA was extracted from DCs before maturation (iDCs, day 10) and after TNFα-induced maturation in the presence or absence of IL-10 (DCs, day 13, ± IL-10). A RT-PCR was carried out in order to assess expression of Notch pathway components (Fig. IV.3A). Expression of Jagged1 and Delta1 was upregulated upon maturation. IL-10 treatment had no visible effect on the TNFα-induced increase of Jagged1 but inhibited upregulation of Delta1. However, in another experiment Delta1 expression did not increase upon maturation of DCs and was further downregulated by the presence of IL-10 (data not shown). Jagged1 was very weakly expressed, whereas Hes1 could not be detected. Expression of Notch1 and Notch2 did not alter greatly between the various treatments.

To more accurately quantify the difference of expression, total RNA was reverse transcribed into cDNA and analysed by real-time PCR (Fig. IV.3B). The upregulation of Jagged1 upon maturation was also very pronounced after amplification by real-time PCR. The presence of IL-10 increased Jagged1 expression further. Delta1 expression did not alter between the different treatments. In a second experiment, Delta1 expression decreased slightly upon maturation but did not reach a 3-fold difference (data not shown). Furthermore, expression levels were very low. Expression of Jagged2, Notch1 and Notch2 did not differ significantly between the different treatments. Hes1 and Hes5 were not detected, whereas Deltex1 was expressed in iDCs and downregulated upon maturation in the presence or absence of IL-10. IL-10 transcripts were generally very low. Upon maturation, a small increase of IL-10 expression was observed. IL-12p70 consists of two chains, a p40 protein and a p35 protein, which are both required for activity (Heufler *et al.*, 1996; Macatonia *et al.*, 1995). Transcripts for both subunits were upregulated upon TNFα-induced maturation. The presence of IL-10 during the maturation process further increased expression of IL-12p35, but decreased IL-12p40 compared to maturation in the absence of IL-10.

TNFα is a very potent maturation stimulus for bone marrow-derived DCs (Lutz *et al.*, 1999; Reid *et al.*, 1992): TNFα-induced maturation increased cell surface expression of adhesion

molecule CD11c, costimulatory molecule B7.2 and MHC class II molecules. The presence of IL-10 during the maturation process had only minimal effect on the expression of these molecules. However, IL-10-treated DCs were less potent stimulators of an allogeneic T cell response *in vitro* and produced decreased amounts of IL-12p40 compared to DCs matured in the absence of IL-10. Interestingly, IL-12p35 was further upregulated by IL-10 compared to DCs matured in the absence of IL-10. Transcripts for Jagged1 were strongly upregulated upon maturation and further increased by the presence of IL-10. There was no evident Notch signalling occurring after 64 hours of maturation as concluded from the absence of downstream targets Hes1 and Hes5 and the downregulation of Deltex1.

These data imply that members of the Notch family are differentially expressed during the maturation process in DCs. It was of interest to investigate in more detail the kinetic of Jagged1 upregulation and the influence of IL-10 on this process. Because of a very poor yield of DCs with the culture method described above, I switched to a different protocol based on Inaba's method (Inaba *et al.*, 1992a).



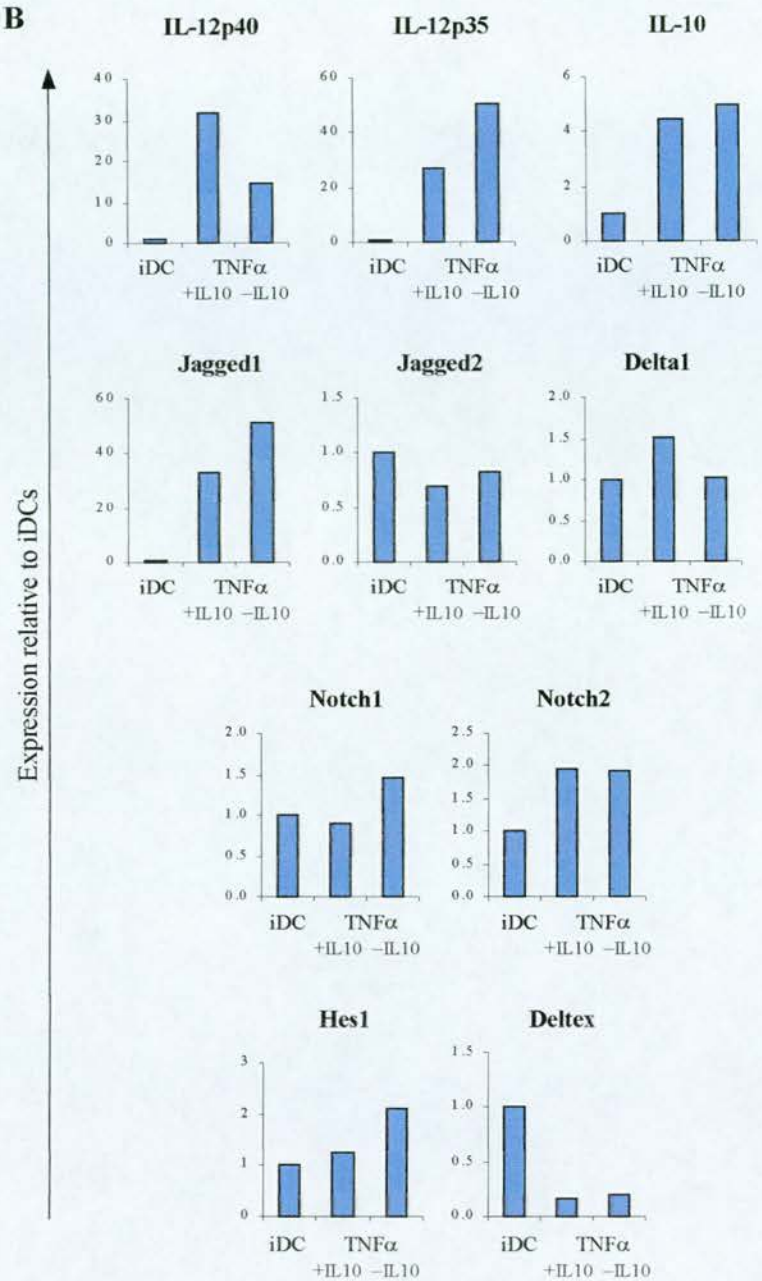


Figure IV.3. Maturation of DCs with TNFα in the presence or absence of IL-10 leads to differential gene expression of Notch pathway components and upregulation of Jagged1 transcript.

Bone marrow-derived DCs from C57BL/6 mice were grown for 10 days and then matured with 50ng/ml TNFα for 48 hrs in the presence or absence of 50ng/ml IL-10 (TNFα +/– IL-10) (Scheicher *et al.*, 1992). Total RNA was extracted and quality was tested by RT-PCR as described in materials & methods, section II.2.16.

[A] The RNA was reverse transcribed and amplified by RT-PCR. The PCR-amplified products were run in a 2% agarose gel. β-Actin was used as a house keeping gene to normalise all the RNA samples to the same amount of starting template and as a control of the PCR reaction itself.

[B] RNA was reverse transcribed to cDNA and amplified by real-time PCR as described in materials & methods, section II.2.21. Levels of mRNA transcripts are shown relative to the levels of the internal calibrator which were immature DCs taken at day 10 prior to maturation (iDC). A change in expression of at least 3-fold or greater was considered significant.

IV.3 Differentiation of bone marrow-derived DCs based on Inaba's protocol

The current protocol was adapted from a previous publication (Inaba *et al.*, 1992a). There exist several differences compared to the previous protocol used, which are outlined in the table IV.1. The main differences in Inaba's method are the shorter culture period, the discarding of cell-containing supernatant during feeding and the use of GM-CSF supernatant instead of recombinant GM-CSF.

Table IV.1. Comparison of DC culture techniques.

Parameter	Method based on Scheicher *et al.*	Method based on Inaba *et al.*
Lyses of red blood cells	Yes	Yes
Starting culture density (BM cells/ml)	$2x10^5$	$3.75x10^5$
Culture plastic	Bacterial quality 100-mm petri dishes	Tissue culture quality 24-well plates
Culture medium	Complete Iscoves	DC-RPMI (= complete RPMI without β-mercaptoethanol)
GM-CSF dose	20ng/ml rmGM-CSF*, from day 6: 10ng/ml rmGM-CSF*	5% murine hybridoma (X63-gmcsf) supernatant[§] throughout
Feeding	Day 3 add 100% of complete RPMI + GM-CSF, days 6 and 8 aspirate 50% supernatant containing cells, wash, resuspend cells in fresh medium + GM-CSF and give back to the petri dish	Day 3 and 6 aspirate and discard 80% supernatant (containing cells), add fresh medium + GM-CSF
Culture period before transfer	10 days	7 days
Culture density for maturation (iDCs/ml)	$2.5x10^5$	10^6
Maturation	By 50ng/ml TNFα ± 50ng/ml IL-10	By 50ng/ml TNFα ± 50ng/ml IL-10 or by 100ng/ml LPS
Maturation period	64 hours	2 – 72 hours

*rmGM-CSF = recombinant murine GM-CSF (R&D Systems)
[§] corresponds to 10-15ng/ml GM-CSF (see Materials & Methods, section II.3.1.5)

IV.3.1 Purity of bone marrow-derived cells at day 7

Although a lot of granulocytes and B lymphocytes were removed during the feeding steps in Inaba's protocol, the shorter culture time and the sustained dose of GM-CSF may have a negative effect on the purity of the iDC population on the day of transfer (day 7). Therefore, the purity of the iDC population was analysed by flow cytometry.

Bone marrow cells from BALB/c mice were cultured in GM-CSF as described in materials & methods, section II.3.1.3. At day 7 of culture, purity of iDC population was analysed by flow cytometry (Fig. IV.4). The cells were not gated to avoid excluding the population of smaller-sized granulocytes and lymphocytes. There were no $CD19^+B220^+$ cells (B lymphocytes), $CD4^+CD3\varepsilon^+$ or $CD8^+CD3\varepsilon^+$ cells ($CD4^+$ or $CD8^+$ T lymphocytes, respectively) detected (Fig. IV.4A). However, almost 20% of cells were positive for Ly-6G (Gr1) (Fig. IV.4B) confirming a contamination with granulocytes. Contamination of macrophages was analysed by staining cells for the macrophage/monocyte markers sialoadhesin (CD169), CD14 and F4/80. Cells were negative for sialoadhesin and CD14. However, a majority of cells expressed intermediate levels of the macrophage marker F4/80.

The iDC population on day 7 of Inaba's protocol is free of lymphocytes, but contains a 20% granulocyte contamination. All cells were negative for macrophage/monocyte markers sialoadhesin and CD14, whereas monocytes and macrophages showed low levels of staining for both markers (data not shown). Over 50% of the cells were F4/80-positive, a macrophage marker, which is also expressed on DCs (Lutz *et al.*, 1999; Mahnke *et al.*, 1997). Therefore, it was concluded that the main contaminants of the iDC population were granulocytes.

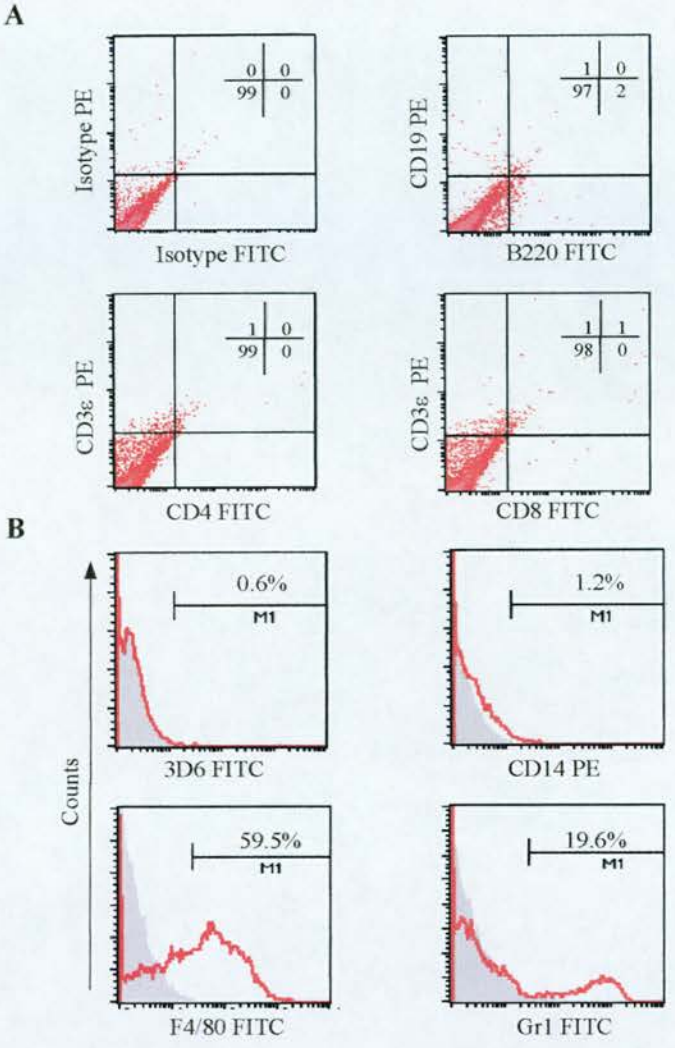


Figure IV.4. Purity of bone marrow-derived cells at day 7.
Bone marrow-derived DCs from BALB/c mice were grown based on a method described by Inaba *et al.* (Inaba *et al.*, 1992a). Immature DCs (iDCs) at day 7 were analysed by flow cytometry.
[A] Dot plots showing ungated cells double stained for B (CD19 and B220) or T cell markers (CD3ε and CD4 or CD8).
[B] Histograms showing ungated cells stained for the macrophage markers 3D6, CD14 and F4/80 and granulocyte marker Gr1. Shaded histogram represents isotype control. Numbers indicate percentage of cells within M1.
Quadrant grid was set using isotype controls. Numbers in the cross within dot plots represent percentages within the corresponding quadrant. Details of antibodies are given in table II.4 & II.5.

IV.3.2 Maturation of bone marrow-derived DCs with TNFα or LPS upregulates costimulatory molecules and activation markers.

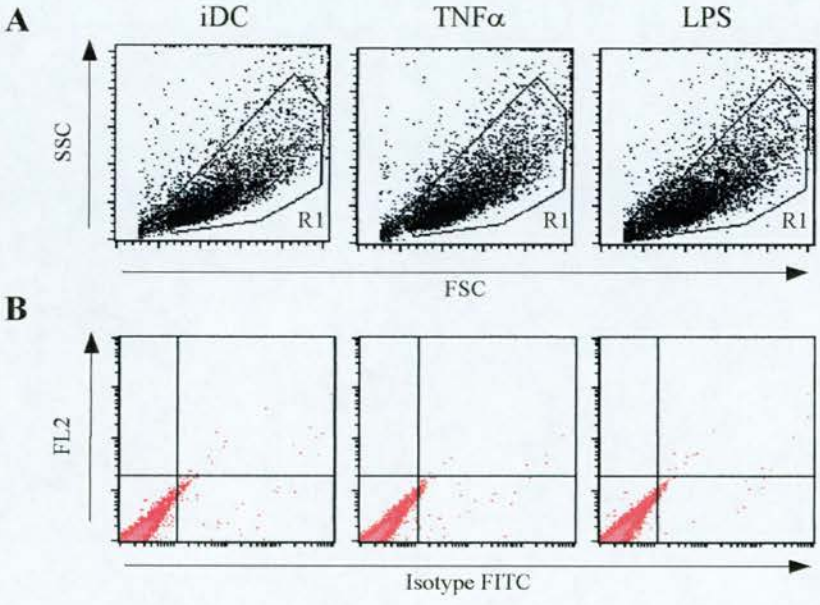
To show that the remaining ~80% of cells were actually immature DCs, their ability to upregulate costimulatory molecules and activation markers upon maturation was tested. In addition to the endogenous maturation stimulus TNFα, DCs were also matured with the microbial stimulus LPS.

Bone marrow cells from BALB/c mice were cultured in GM-CSF as above. At day 7 of culture, an aliquot of the non-adherent immature DCs (iDCs) were kept for analysis by flow cytometry and the rest was transferred into a 24-well plate for maturation with TNFα or LPS. DCs were analysed by flow cytometry after 24 hours (Fig. IV.5). Cells were double stained for MHC class II plus costimulatory molecules B7.1, B7.2 and CD40 and adhesion molecules ICAM-1 and CD11c. Percentages of cells staining positive for these markers were summarised in table IV.2. Around 70 to 80% of the iDCs and matured DCs expressed MHC class II molecules on the cell surface, which is consistent with the previously measured 20% of granulocyte contamination. Even though percentages of MHC class II-, B7.1- and ICAM-1-positive cells did not increase greatly, a visible shift to increased expression was observed on the dot plots. The population of MHC class II^{low} $B7.2^{negative}$ cells (lower right quadrant) present in the iDC sample decreased in TNFα-treated DCs and almost completely disappeared in LPS-matured DCs. Instead a distinct population of MHC class II^{high} $B7.2^{high}$ cells appeared in TNFα- and LPS-matured DCs. In this particular experiment, TNFα only marginally upregulated expression of CD40, whereas LPS induced moderate CD40 expression. In other experiments, TNFα induced low, whereas LPS induced high expression of CD40 (Table IV.2). In contrast to earlier observations (IV.2.1) expression of CD11c decreased upon maturation.

Here it has been shown that both stimuli, TNFα and LPS increased expression of MHC class II molecules, costimulatory molecules B7.1 and B7.2 and adhesion molecule ICAM-1. However, only LPS induced expression of CD40. Adhesion molecule CD11c was downregulated upon maturation.

Table IV.2. Maturation of DCs upregulates costimulatory molecules and activation markers. Percentage of cells stained positive for the indicated markers of experiment IV.5 and the mean and standard deviation (SD) of four independent experiments.

Cell surface marker	iDC		TNFα		LPS	
(%)	Fig. IV.5	Mean ± SD	Fig. IV.5	Mean ± SD	Fig. IV.5	Mean ± SD
MHC class II	70	72 ± 6	77	80 ± 6	69	76 ± 8
B7.1	65	67 ± 8	82	86 ± 6	79	84 ± 8
B7.2	33	38 ± 6	64	67 ± 10	67	72 ± 10
ICAM-1	82	85 ± 3	97	98 ± 2	93	96 ± 3
CD40	2	1 ± 1	4	26 ± 19	23	46 ± 18
CD11c	47	51 ± 5	37	39 ± 10	26	32 ± 23



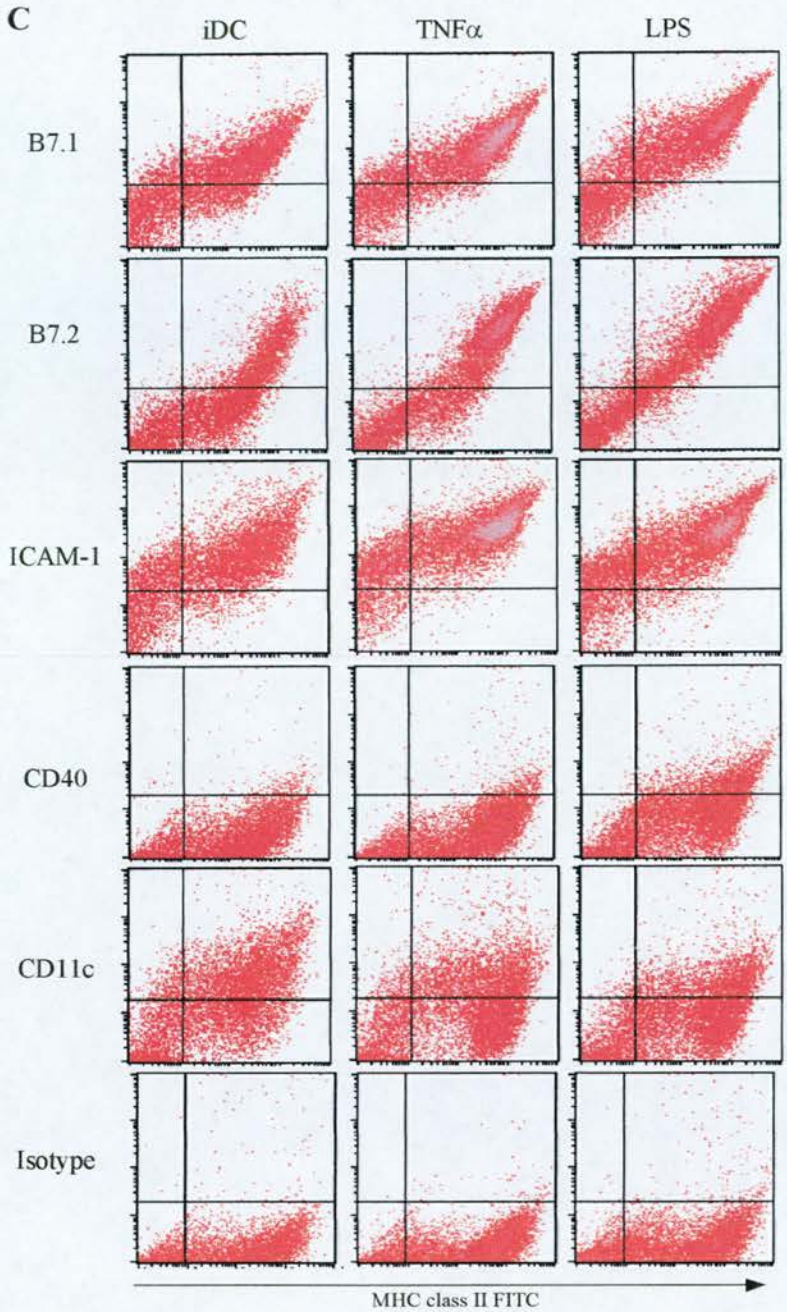


Figure IV.5. Maturation of bone marrow-derived DCs with TNFα or LPS upregulates costimulatory molecules and activation markers.
Bone marrow-derived DCs from BALB/c mice were grown based on a method described by Inaba *et al.* (1992a) (iDC) and then matured with 50ng/ml TNFα (TNFα) or 100ng/ml LPS (LPS) for 24 hrs. The cells were double stained for MHC class II and costimulatory molecules B7.1, B7.2, CD40, adhesion molecules ICAM-1 and CD11c and analysed by flow cytometry.
[A] Forward (FSC) and side scatter (SSC) plots showing the R1 gate for live cells used in subsequent analysis.
[B] Dot plot analysis showing the FITC-conjugated isotype control for the anti-MHC class II antibody (FL1).
[C] Dot plot analysis with PE-conjugated antibodies (FL2) and FITC-conjugated anti-MHC class II antibody (FL1). Quadrant grid was set using isotype controls. Details of antibodies are given in table II.4 & II.5.

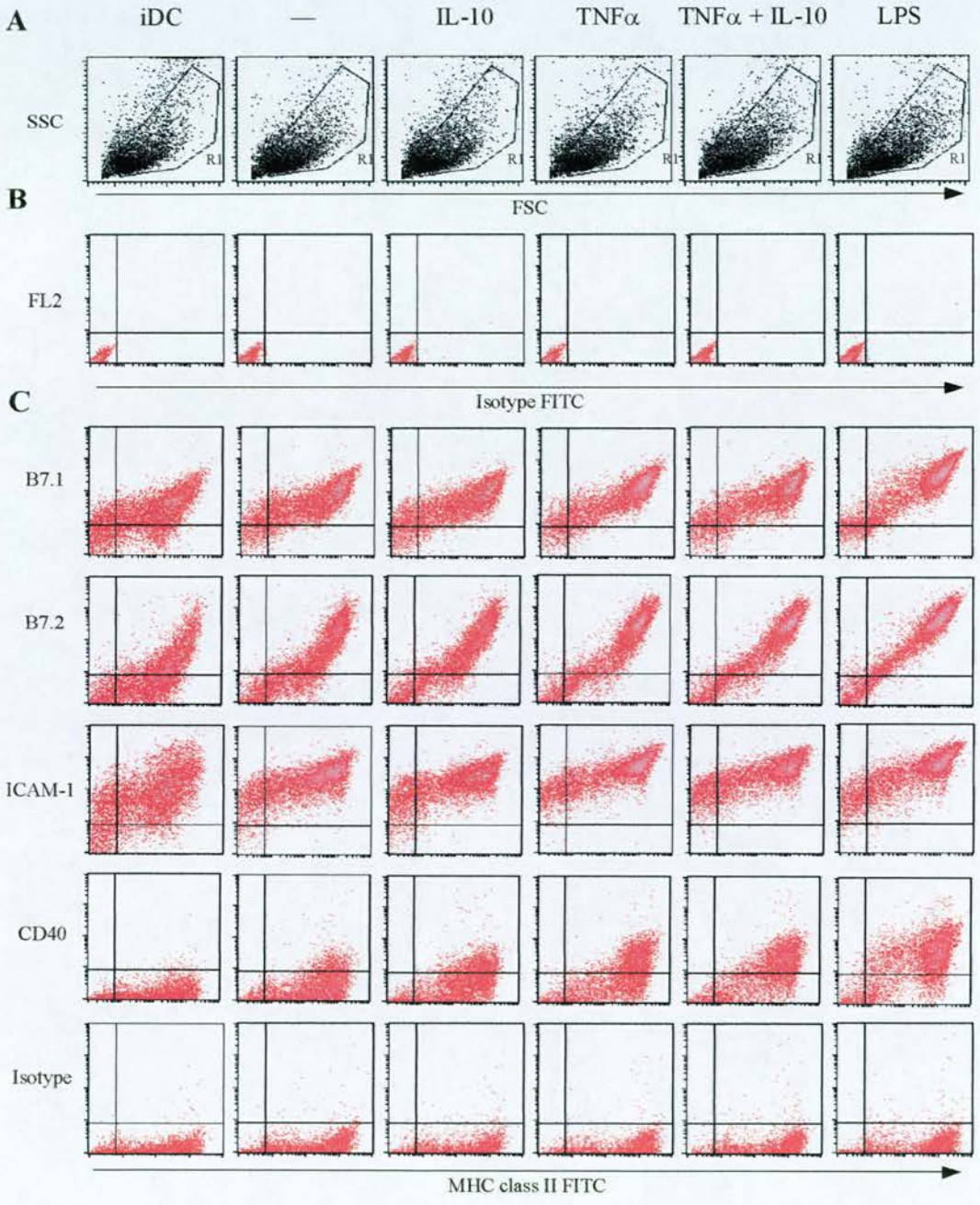
IV.3.3 Maturation of DCs in the presence of IL-10 induces downregulation of most costimulatory molecules and activation markers after 24 hours

Here, the influence of IL-10 and the kinetic of the upregulation of costimulatory molecules were investigated. Bone marrow-derived DCs were grown as described above. At day 7 an aliquot of iDCs was kept for flow cytometry. The remaining cells were transferred to new wells and grown in medium ± IL-10 as a control for spontaneous maturation induced by the transfer, in TNFα ± IL-10 and in LPS. After 2, 8, 16, 24 and 48 hours, cells were analysed by flow cytometry. At the 72 hour time point, only TNFα-matured cells were analysed. To exclude interference of dead cells with the analysis, the viability dye 7-aminoactinomycin D (7-AAD) staining necrotic and late apoptotic cells was added to the sample before analysis. Cells were double stained for MHC class II molecules and B7.1, B7.2, ICAM-1 or CD40. Staining for CD11c was omitted because this adhesion molecule did not indicate maturation of DCs using Inaba's method (Table IV.2). Figure IV.6 A-C shows dot plot analysis of iDCs and cells grown for 24 hours after the transfer as a representative example. The geometrical mean fluorescence of MHC class II molecules, B7.1, B7.2 and ICAM-1 of class II-positive cells (gated on upper and lower right quadrants in figure IV.6C) is shown in figure IV.6D. Percentage of CD40-positive cells and of the cell population expressing high amounts of class II and B7.2 as defined in figure IV.6F is summarised in IV.6E.

Transfer of iDCs into new wells on day 7 of culture induced moderate maturation, as seen by upregulation of activation markers and by the increase of class II^{high} $B7.2^{high}$ cells. Interestingly, addition of IL-10 slightly increased expression of CD40. Even though the increase was small, it has been observed reproducibly. Maturation of DCs with LPS rapidly upregulated class II, B7.1, B7.2 and ICAM-1. However, the increased expression was only transient and all activation markers except B7.1 were downregulated after 20 hours of maturation. Maturation with TNFα was a much slower process. Maximal expression of the above mentioned activation markers were observed at 72 hours. Addition of IL-10 during maturation with TNFα decreased expression of the activation markers only at 24 hours or later. Interestingly, the presence of IL-10 during TNFα-induced maturation enhanced expression of B7.2 and CD40 within the first 24 hours.

Looking at class II^{high} $B7.2^{high}$ cells (Fig. IV.6F) summarises the observations made with the different activation markers (Fig. IV.6E): Maturation induced by LPS was much quicker, but

only transient, whereas TNFα induced maturation of DCs with a sustained phenotype of mature DCs. Addition of IL-10 slightly accelerated maturation of DCs within the first 24 hours before it induced a general decrease in activation markers. Transfer of iDCs at day 7 induced some degree of activation in the absence of any maturation stimuli as seen by the increase of class II^{high} $B7.2^{high}$-positive cells.



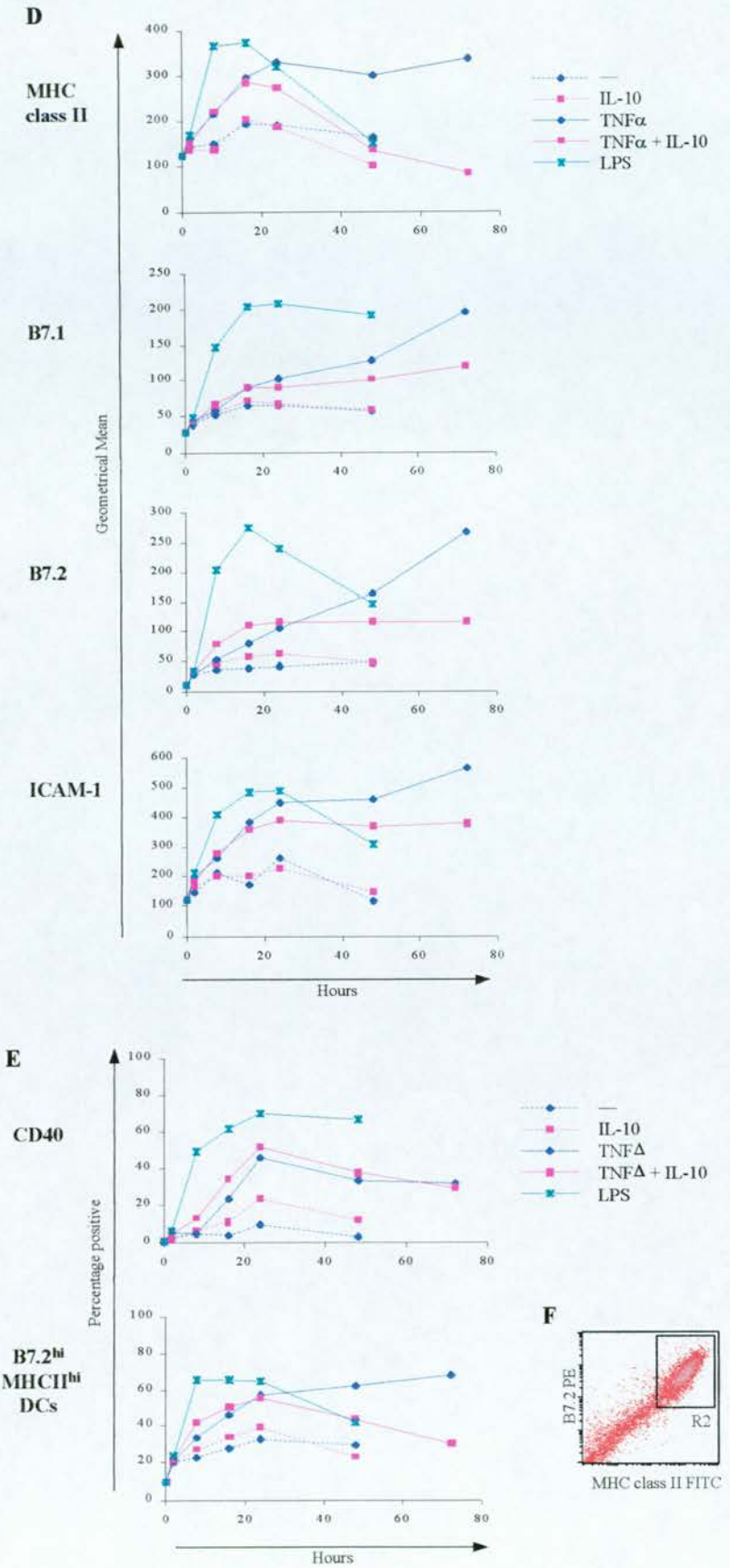

D
MHC class II
B7.1
B7.2
ICAM-1
Geometrical Mean
Hours
—
IL-10
TNFα
TNFα + IL-10
LPS
E
CD40
B7.2hi MHCIIhi DCs
Percentage positive
Hours
—
IL-10
TNFΔ
TNFΔ + IL-10
LPS
F
B7.2 PE
R2
MHC class II FITC

Figure IV.6. Maturation of DCs in the presence of IL-10 induces downregulation of most costimulatory molecules and activation markers after 24 hours.
Bone marrow-derived DCs from BALB/c mice were grown based on a method described by Inaba *et al.* (1992a). At day 7, iDCs were either incubated in medium alone (—) or in 50ng/ml IL-10 (IL-10) or matured with 50ng/ml TNFα in the presence or absence of IL-10 (TNFα or TNFα + IL-10) or with 100ng/ml LPS (LPS). At 0, 2, 8, 16, 24, 48 or 72 hrs, cells were double stained for MHC class II and B7.1, B7.2, CD40 or ICAM-1 and analysed by flow cytometry. Dead cells were excluded by uptake of 7-AAD. Details of antibodies are given in table II.4 & II.5.
[A]-[C], Flow cytometry analysis iDCs and of cells taken at 24 hrs are shown as a representative.
[A] Forward (FSC) and side scatter (SSC) plots showing the R1 gate for live cells used in subsequent analysis.
[B] Dot plot analysis showing the FITC-conjugated isotype control for the anti-MHC class II antibody (FL1).
[C] Dot plot analysis with PE-conjugated antibodies (FL2) and FITC-conjugated anti-MHC class II antibody (FL1). Quadrant grid was set using isotype controls.
[D] Geometrical mean fluorescence for MHC class II, B7.1, B7.2 and ICAM-1 of MHC class II-positive cells plotted against maturation time.
[E] Percentage of CD40-positive DCs or $B7.2^{hi}$ $MHCII^{hi}$ DCs (defined in figure F) plotted against maturation time.
[F] Dot plot analysis with PE-conjugated anti-B7.2 (FL2) and FITC-conjugated anti-MHC class II antibody (FL1). Region R2 selects for cells expressing $B7.2^{hi}$ and $MHCII^{hi}$

IV.3.4 Downregulation of costimulatory molecules and activation markers in the presence of IL-10 is not due to increased cell death

The decrease in activation markers seen in DCs matured in the presence of IL-10 at later time points may be due to the onset of apoptosis. It has been shown that IL-10 reduced viability of human Langerhans cells (Ludewig *et al.*, 1995).

Therefore the amount of dead cells was monitored during the maturation process by staining with Annexin V and 7-AAD. Annexin V detects early and late apoptotic and necrotic cells whereas 7-AAD only stains late apoptotic and necrotic cells (Vermes *et al.*, 1995). Therefore, cells showing Annexin V and 7-AAD staining were referred to as dead cells since distinction between apoptosis and necrosis is not possible. Bone marrow cells were cultured and matured as described above and analysed by flow cytometry for Annexin V and 7-AAD staining.

Again, iDCs and DCs cultured for 24 hours after transfer were shown as a representative (Fig. IV.7A). Percentage of Annexin V and 7-AAD double-positive cells over the time course are shown in figure IV.7C. Only in the LPS-treated culture an increase of dead cells was observed after 24 hours of maturation. In all other cultures, percentage of dead cells did not exceed 10%.

Therefore, I concluded that the decreased expression of activation markers in DCs matured in the presence of IL-10 was not due to increased cell death.

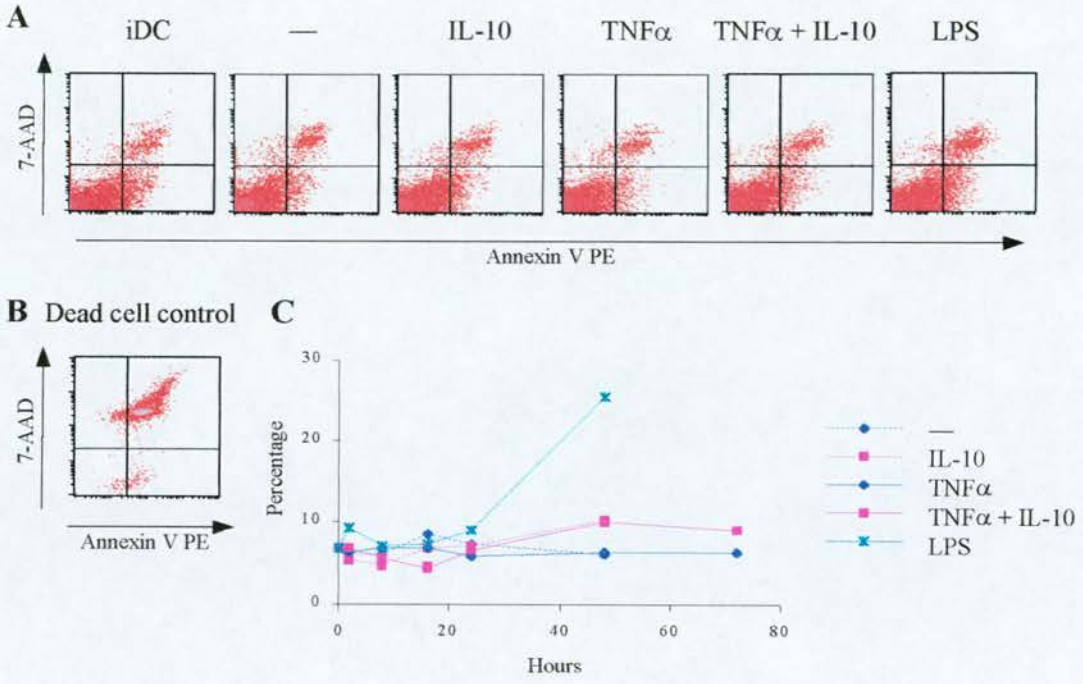


Figure IV.7. Downregulation of costimulatory molecules and activation markers in the presence of IL-10 is not due to increased cell death.
Bone marrow-derived DCs from BALB/c mice were matured and stained for Annexin V at the same time points as in figure IV.6. 7-AAD was added prior to flow cytometry analysis. As a positive control for Annexin V and 7-AAD staining, an aliquot of DCs were incubated at 95°C for 2 mins (dead cell control).
[A] Dot plot analysis with PE-conjugated Annexin V (FL2) and 7-AAD (FL3) of cells taken at 24 hrs were shown as a representative.
[B] Dead cell control.
[C] Percentage of Annexin V and 7-AAD double-positive cells plotted against maturation time.

IV.3.5 Immature DCs and DCs matured in the presence of IL-10 are less potent stimulators of allogeneic T cell proliferation than those matured in the absence of IL-10.

Human IL-10 modulated DCs from peripheral blood induce an antigen-specific anergy of $CD4^+$ and $CD8^+$ T cells (Steinbrink *et al.*, 1999; Steinbrink *et al.*, 1997). This state of anergy was characterised by inhibited T cell proliferation and reduced production of IL-2 and IFNγ. The stimulatory capacity of DCs matured in the presence of IL-10 was examined by carrying out a MLR. Bone marrow-derived DCs from BALB/c mice ($H\text{-}2^d$) were grown and matured for 24 hours as previously described and used in a MLR with $CD4^+$ T cells purified from spleens of C57BL/6 mice by MACS ($H\text{-}2^b$). After 4 days proliferation was measured by [^{3}H]thymidine uptake (Fig. IV.8). DCs incubated in medium alone or in IL-10 induced moderate T cell proliferation. Increase of proliferation by TNFα-matured DCs was small but significant ($P<0.05$), whereas LPS-treated DCs induced strong T cell activation ($P<0.001$). Addition of IL-10 during TNFα-mediated maturation of DCs inhibited the stimulatory capacity significantly ($P<0.001$).

LPS-matured DCs induced a stronger proliferative response in $CD4^+$ T cells than TNFα-matured DCs. The addition of IL-10 during the maturation with TNFα reduced the capability of the DC to activate $CD4^+$ T cells.

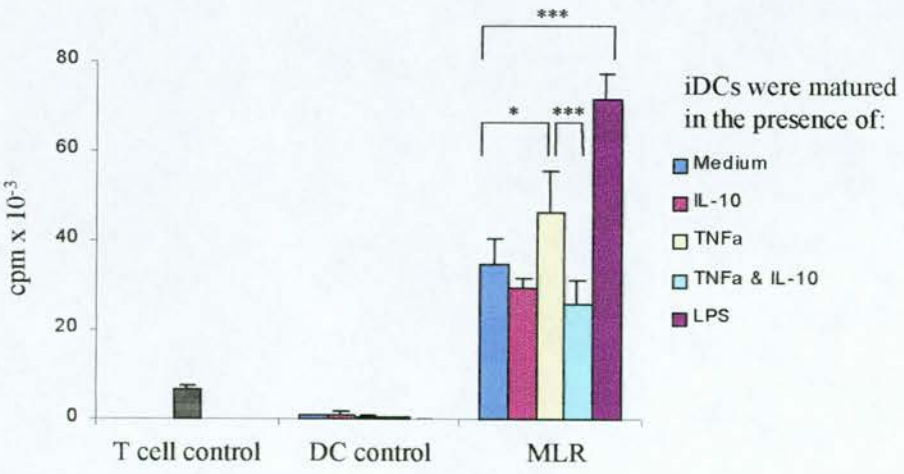


Figure IV.8. Immature DCs and DCs matured in the presence of IL-10 are less potent stimulators of allogeneic T cell proliferation than those matured in the absence of IL-10.
Bone marrow-derived DCs from BALB/c mice were matured for 24 hrs as described in figure IV.6. A MLR was carried out with DCs as stimulators (10^4/well) and MACS-purified $CD4^+$ T cells from C57BL/6 spleens ($2x10^5$/well). After 4 days, proliferation was measured by $[^3H]$thymidine uptake during the last 18 hrs of culture and plotted as cpm x 10^{-3} ± standard deviation. * : $P<0.05$; *** : $P<0.001$ statistical comparison of sample data was performed by application of ANOVA Test using the GraphPad InStat programme (GraphPad Software Inc., www.graphpad.com).

IV.3.6 Maturation of DCs with TNFα in the presence of IL-10 blocks IL-12p40 induction.

Many groups have reported a decrease in IL-12 production if DCs were matured in the presence of IL-10 (Bellinghausen *et al.*, 2001; Brossart *et al.*, 2000; Buelens *et al.*, 1997; Koch *et al.*, 1996; Takenaka *et al.*, 1997). Therefore, the amount of heterodimeric IL-12p70 and the IL-12p40 subunit was measured in the supernatant of DC matured as described in figure IV.6 as an additional read-out for IL-10-induced inhibition of DC maturation. Additionally, production of IL-10 was measured in DC incubated in medium alone or matured with TNFα or LPS.

IL-12p70 was only detected in the cultures of LPS-matured DCs (Fig. IV.9A). The subunit p40 was strongly induced by LPS (Fig. IV.9B) and to a much lesser extent with TNFα (Fig. IV.9C). However, IL-10 abrogated TNFα-induced production of IL-12p40. Two hours after transfer of iDCs, low amounts of IL-10 were detected in untreated, LPS- and TNFα-activated DCs (Fig. IV.9D). Whereas LPS further induced production of IL-10, amount of IL-10 decreased in cultures of untreated and TNFα-treated DCs.

LPS- but not TNFα-induced maturation of DCs induced production of IL-12p70 and IL-10. Addition of exogenous IL-10 to TNFα-activated DCs suppressed the release of the IL-12p40 subunit.

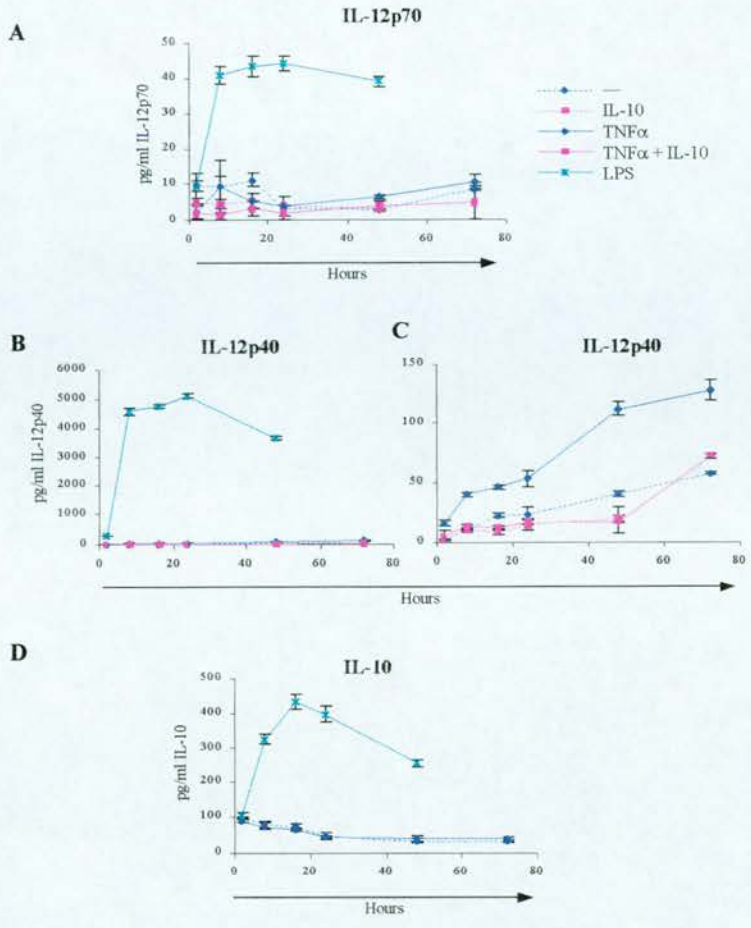


Figure IV.9. Maturation of DCs with TNFα in the presence of IL-10 blocks IL-12p40 induction. Bone marrow-derived DCs from BALB/c mice grown and matured as described in figure IV.6. After 0, 2, 8, 16, 24, 48 and 72 hrs, IL-12p70 [A], IL-12p40 ([B] and [C], the latter without the data for LPS-matured DCs) and IL-10 [D] were measured in the supernatant by ELISA.

IV.3.7 Depletion of granulocytes does not activate DCs.

Having ensured that DCs were generated by Inaba's method, that they can be activated by TNFα or LPS and that maturation in the presence of IL-10 led to observations described by others, gene expression of components of the Notch pathways was examined. However, in contrast to Scheicher's method, the DC generated by Inaba's protocol contained high contamination of granulocytes (Fig. IV.4). Using paramagnetic beads, a depletion protocol was developed to deplete granulocytes prior to RNA analysis.

It is important to examine whether the depletion had any immediate effects on the maturation state of the DCs. Therefore, iDCs were stained for MHC class II molecules and B7.2 before

and after depletion (Fig. IV.10A). To control the efficiency of the depletion, cells were stained for the granulocyte marker Gr1. The anti-Gr1 antibody used for flow staining and depletion originates from the same clone. Hence it cannot be used to examine the efficiency of depletion because the anti-Gr1 antibody used for depletion may mask Gr1 molecules on non-depleted granulocytes. To circumvent this problem, an indirect staining protocol was developed, whereby the unconjugated depletion antibody was used as the primary antibody for flow staining. FITC-conjugated anti-rat IgG antibody was used to detect remaining granulocytes after the depletion process. The depletion reduced contamination of granulocytes from 33 to 9% without activating the DCs as demonstrated by the unchanged expression of class II molecules and B7.2 (Fig. IV.10A). The depletion of granulocytes can also be observed by the increase of class II^+ and $B7.2^+$ cells in figure IV.10A and the decreased population of class II^- $B7.2^-$ cells in figure IV.10B. After 48 hours of maturation with LPS, depletion of granulocytes led to 95% class II- B7.2-positive cells suggesting that contamination of was less than 5%. Viability of DCs was not affected by the depletion (data not shown).

Using Dynabeads to deplete contaminating granulocytes led to an over 90% pure population of class II^+ $B7.2^+$ cells. The depletion did not affect the maturation states of the DCs as judged by the unaltered cell surface phenotype.

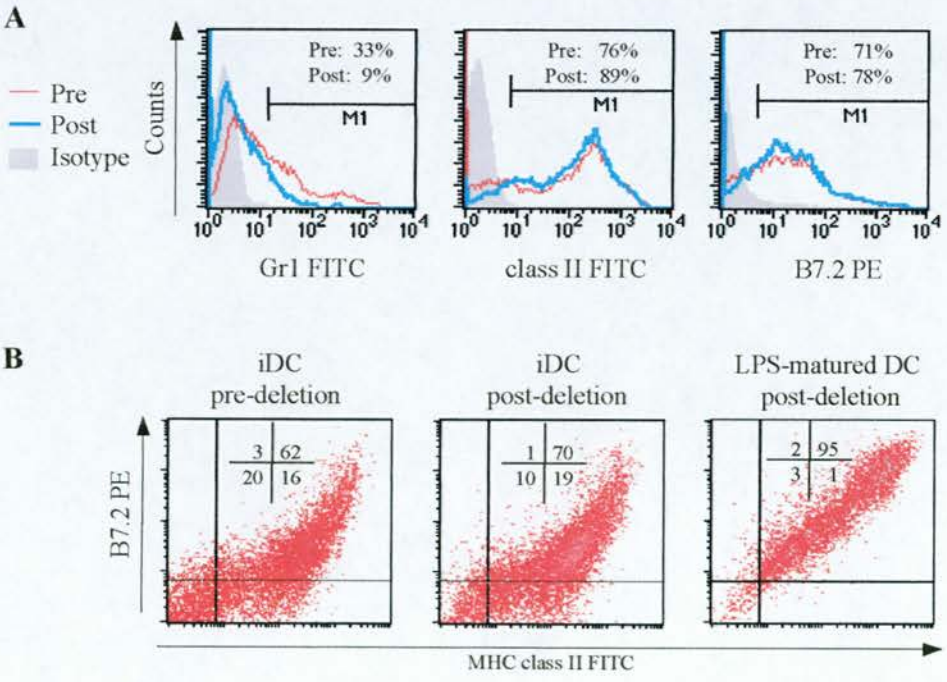


Figure IV.10. Depletion of granulocytes does not activate DCs.
Bone marrow-derived DCs from BALB/c mice were grown as in figure IV.4. At day 7, iDCs were stained indirectly for Gr1 (1° antibody: rat anti-Gr1, 2° antibody: FITC-conjugated anti-rat IgG) or double stained for class II and B7.2 before (pre) and after depletion (post) of contaminating granulocytes using Dynabeads (materials & methods II.3.1.4). An aliquot of cells was matured with 100ng/ml LPS for 48 hrs (LPS) and then depleted and stained for flow cytometry. Dead cells were excluded by uptake of 7-AAD. Details of antibodies are given in table II.4 & II.5.
[A] Histograms showing iDCs stained for the indicated cell surface molecules before (pre) and after (post) depletion. Shaded histogram represents isotype control. Numbers indicate percentage of cells within M1.
[B] Dot plot analysis with FITC-conjugated anti-MHC class II antibody and PE-conjugated anti-B7.2 antibody. Quadrant grid was set using isotype controls. Numbers in the cross within dot plots represent percentages within the corresponding quadrant.

IV.3.8 Maturation of DCs in the presence or absence of IL-10 leads to differential gene expression of Notch ligands Jagged1 and Delta1.

Using the depletion protocol described above, gene expression of iDCs grown according to Inaba's method and DCs matured in medium ± IL-10, TNFα ± IL-10 or in LPS was analysed. Prior to RNA extraction, granulocytes were depleted. Total RNA was reverse transcribed into cDNA and analysed by real-time PCR (Fig. IV.11). IL-12p40 transcripts were strongly induced in DCs matured with LPS and to a much lesser extent in DCs matured with TNFα. Interestingly, TNFα induced an early peak followed by a later rise of p40 expression. The presence of IL-10 inhibited TNFα-induced p40 expression. In contrast to IL-12 subunit p40, p35 was mainly induced after 24 hours of maturation with LPS. Interestingly, the presence of IL-10 increased transcription levels of p35 in TNFα-matured DCs. Transcription of IL-10 was increased in DCs incubated in IL-10 or in TNFα. However, in other experiments where non-depleted DCs were analysed, LPS but not TNFα induced IL-10 expression (data not shown). Maturation of DCs induced expression of Jagged1. Whereas the presence of IL-10 alone did not alter level of Jagged1 transcripts, it increased and prolonged the upregulation in combination with TNFα. Expression of Jagged2 did not change between the different

treatments, whereas expression of Delta3 was not detected. Expression of Delta1 was in all samples very low, even though it was upregulated by IL-10 and by TNFα at early time points or late time points, respectively. However, this upregulation was not reproducible: In a second experiment, only TNFα but not IL-10 induced a transient upregulation, whereas LPS but not TNFα increased Delta1 transcripts in a third experiment (data not shown). Interestingly, an increase in Delta1 expression within the first 24 hours after transfer of iDCs correlated with the upregulation of IL-10 transcripts. Downstream components of Notch signalling were either not detected (Hes5 and Deltex1) or expression did not alter between the different treatments (Hes1). Expression of Notch receptors were not further analysed because they were not differentially regulated in DCs grown according to Scheicher's protocol and neither in preliminary experiments with DCs cultured based on Inaba's method.

Transcript expression of IL-12p40 in TNFα- and LPS-matured DCs correlated with the protein level measured by ELISA (Fig. IV.9). IL-12p35 was induced in matured DCs. The presence of IL-10 during TNFα-induced maturation further increased transcription level. Maturation with TNFα or LPS induced Jagged1 transcription. IL-10 increased and prolonged Jagged1 expression induced by TNFα. Target of Notch signalling (Hes1, Hes5 and Deltex1) were not differentially expressed during maturation.

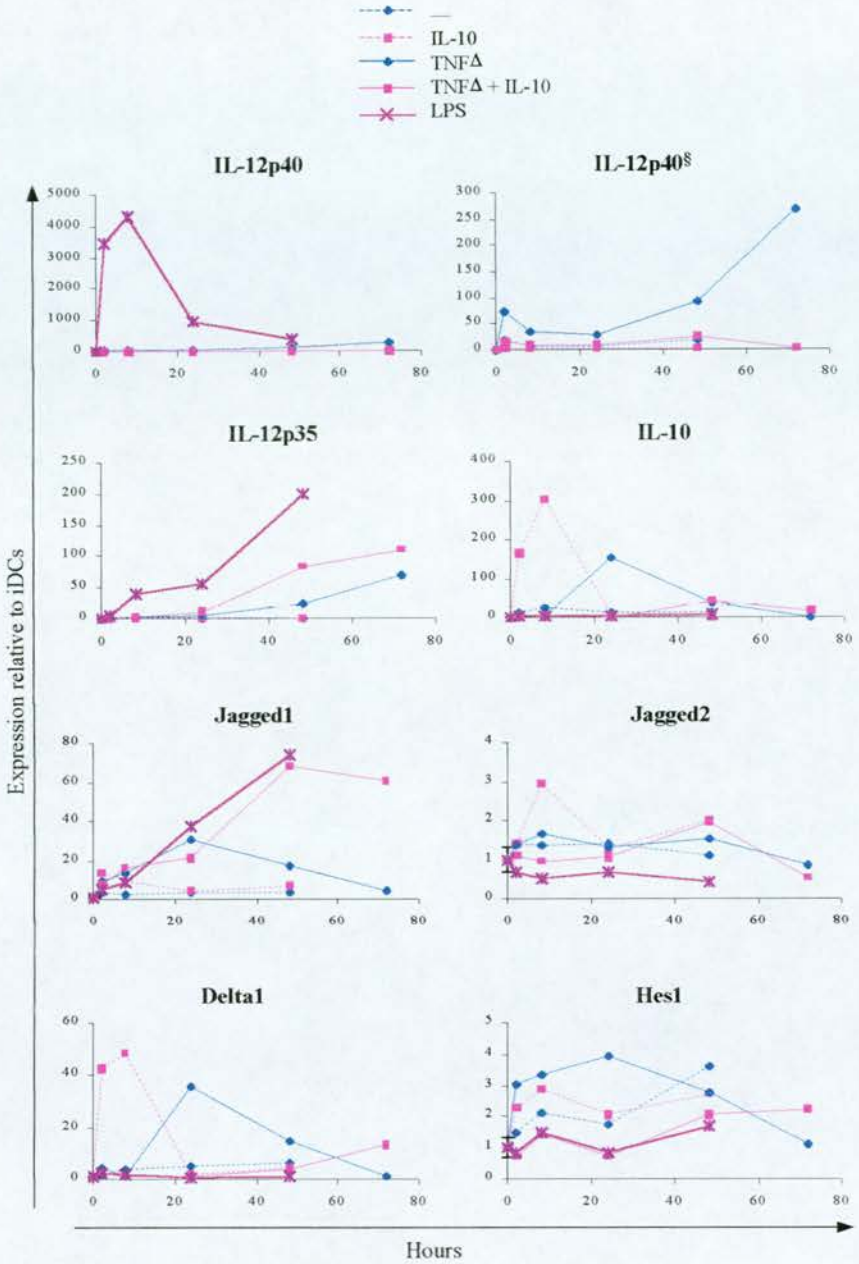


Figure IV.11. Maturation of DCs in the presence or absence of IL-10 leads to differential gene expression of Notch ligands Jagged1 and Delta1.

Bone marrow-derived DCs from BALB/c mice grown and matured as described in figure IV.6. After 0, 2, 8, 16, 24, 48 and 72 hrs, granulocytes were depleted using Dynabeads and total RNA was extracted. RNA was reverse transcribed to cDNA and amplified by real-time PCR as described in materials & methods, section section II.2.21. Levels of mRNA transcripts are shown relative to the levels of the internal calibrator which were immature DCs taken at day 7 prior to maturation (iDC). A change in expression of at least 3-fold or greater was considered significant.

[§] IL-12p40 without the data for LPS-matured DCs

IV.4 Discussion

DCs play a central role in the induction of immune responses. Their ability to prime naïve $CD4^+$ and $CD8^+$ T cells constitutes a unique and critical function of DCs both *in vitro* and *in vivo*. Although it is clear that different DC subpopulations exhibit distinct functions, there is evidence that their functions can also be altered by the cytokine environment. For example, IL-10 has been described to confer a tolerance inducing phenotype in DCs. The immunosuppressive properties of IL-10 on DCs are caused by a reduction in the upregulation of expression of MHC class II molecules and costimulatory and adhesion molecules (Buelens *et al.*, 1995; Sato *et al.*, 1999; Steinbrink *et al.*, 1997). Additionally, IL-10 inhibited production of inflammatory cytokines (e.g. IL-1β, IL-6 and TNFα) and IL-12 (Buelens *et al.*, 1997; Koch *et al.*, 1996; Sato *et al.*, 1999). These altered properties were only seen if IL-10 was added to immature IL-10-sensitive DCs, indicating that IL-10 modulates the function of immature DCs and inhibited their terminal differentiation whereas mature DCs are resistant to IL-10 (Steinbrink *et al.*, 1999; Steinbrink *et al.*, 1997).

Here I propose that the effect of IL-10 is not simply to inhibit maturation of DCs. The presence of IL-10 during TNFα-mediated maturation induced an initial increase of B7.2, CD40 and of class II^{high} $B7.2^{high}$ DCs (Fig. IV.6). Furthermore, IL-10 increased these markers within the first 24 hours also in the absence of TNFα. After 24 hours of maturation, IL-10-treated DCs adopted a tolerance inducing phenotype with decreased expression of costimulatory and adhesion molecules (Buelens *et al.*, 1995; Sato *et al.*, 1999; Steinbrink *et al.*, 1997). The effect of IL-10 was even more pronounced during LPS-mediated maturation (G. Perona-Wright, personal communication). DCs matured more rapidly in the presence of LPS in comparison to TNFα (Fig. IV.6). Consistent with this observation, the addition of IL-10 led to a shorter and more pronounced upregulation of MHC class II molecules and B7.2. 18-24 hours after initiation of maturation, IL-10-treated DCs had downregulated expression of these cell surface proteins. This initial upregulation of activation markers does not contradict published data: Sato, Buelens and Steinbrink *et al.* described the "tolerogenic" DC phenotype only 2 to 4 days after addition of IL-10 (Buelens *et al.*, 1995; Sato *et al.*, 1999; Steinbrink *et al.*, 1997). Furthermore, CD40 ligation in the presence of IL-10 did not result in decreased expression of the human DC-specific activation marker CD83 and of B7.2 after 72

hours of maturation (Buelens *et al.*, 1995). This implies differential effects of IL-10 on different maturation signals.

Bioactive IL-12p70 is composed of p40 and p35 subunits and induced largely in macrophages and DCs by transcriptional induction of the p40 subunit (reviewed in Gately *et al.*, 1998). Microbial stimuli such as LPS and the interaction of mature DCs with $CD4^+$ T lymphocytes involving CD40-CD40 ligand (CD40L) activation induce p40 transcription (Cella *et al.*, 1996; Trinchieri, 1995). TNFα is generally not thought to be an inducer of IL-12 (Cella *et al.*, 1996), however, low level of IL-12p40 was induced in TNFα-matured DCs (Brunner *et al.*, 2000). The presence of IL-10 blocks TNFα-, LPS- and CD40-mediated p40 induction in DCs (G. Perona-Wright, personal communication; Aste-Amezaga *et al.*, 1998; Takenaka *et al.*, 1997). Whereas IL-10 also blocked expression of IL-12p35 in LPS-matured DCs or monocytes (G. Perona-Wright, personal communication; Aste-Amezaga *et al.*, 1998), it increased p35 transcription in TNFα-matured DCs.

In summary, IL-10 mediates not just a blockage of the maturation-induced upregulation of activation markers. In contrast to immature DCs, which were shown to induce regulatory T cells with a $CD4^+CD25^+$ phenotype (Jonuleit *et al.*, 2000), IL-10-treated DCs induced anergy in $CD4^+$ and $CD8^+$ T cells (Steinbrink *et al.*, 1999; Steinbrink *et al.*, 1997). Therefore, iDCs and IL-10-treated DCs will need to possess different properties to be able to induce different T cell responses.

In the thymus, Notch ligands are thought to contribute to the differentiation of thymocytes into αβ vs. γδ and $CD8^+$ vs. $CD4^+$ T lymphocytes (Chang *et al.*, 2000; Kronenberg *et al.*, 2000; Robey *et al.*, 1996; Washburn *et al.*, 1997), although contradicting data exist (Deftos *et al.*, 1998; Deftos *et al.*, 2000; Izon *et al.*, 2001). Jagged1 expressed on APCs has been implicated in the generation of regulatory $CD4^+$ T cells *in vivo* (Hoyne *et al.*, 2000). It was therefore of interest to investigate whether Notch ligands were expressed by DCs. Gene expression of bone marrow-derived iDCs, IL-10-treated and TNFα/LPS-matured DCs was analysed. Jagged1 was strongly upregulated upon maturation (Fig. IV.3 & 11). An increase in Jagged1 expression was also observed in macrophages cultured in the presence of TNFα (data not shown by Nomaguchi *et al.*, 2001). The presence of IL-10 during TNFα-mediated maturation further increased levels of Jagged1 transcripts. However, the amount of Jagged1 transcripts induced by TNFα & IL-10 did not exceed LPS-mediated Jagged1 expression. This suggests that Jagged1 is not directly involved in the induction of anergic T cells by DCs. Furthermore,

iDCs, which express only low levels of Jagged1, can induce regulatory T cells (Jonuleit *et al.*, 2000).

Expression of Jagged2 did not change upon maturation or IL-10 treatment, whereas Delta3 was below detection levels. Similar results were obtained with BM-derived macrophages where expression of Jagged2 or Delta1 was "seldom affected by the stimuli" (data not shown by Nomaguchi *et al.*, 2001). Analysis of Delta1 expression during maturation of DCs produced variable results. This was not due to technical problems using real-time PCR because results obtained by conventional RT-PCR matched the real-time PCR results. Additionally, transfection of a murine fibroblast cell line (L cells) with Delta1 led to an over 3000-fold increase of Delta1 transcripts measured by real-time PCR, whereas transfection with Jagged1 did not alter Delta1 expression and thereby ensuring the specificity of the Delta1 primers and probe (Fig. V.3). Generally, DCs expressed very little Delta1 as observed by high C_T values. Interestingly, upregulation of Delta1 in the first 24 hours under certain conditions correlated with upregulation of IL-10 within the same experiment. RNA analysis of DCs, which have not been depleted of granulocytes, showed that LPS- but not TNFα-treatment upregulated transcripts for Delta1 and IL-10 in DCs (data not shown). This observation was consistent with the detection of IL-10 in culture medium of LPS-matured DCs, which has been described previously (Langenkamp *et al.*, 2000).

IL-10 can be expressed by a wide range of cell types including T cells, B cells, monocytes, macrophages, keratinocytes and many tumour cells (de Waal Malefyt *et al.*, 1991; Del Prete *et al.*, 1993; Enk and Katz, 1992; Groux *et al.*, 1997; O'Garra *et al.*, 1992). A few groups also reported granulocytes to produce IL-10 (Romani *et al.*, 1997; Shimonkevitz *et al.*, 2000). Since granulocyte-depleted DCs did not increase IL-10 transcripts after LPS activation, I cannot exclude that the granulocytes were the major group of IL-10 producing cells in this culture.

Notch receptors were not differentially expressed depending on the activation status of the DCs. Neither were there any signs of Notch signalling: Transcripts for Hes5 were not detected in DCs grown using the culture conditions described by either Scheicher or Inaba. Even though Hes1 was expressed in DCs, it was not differentially modulated during activation of DCs. Deltex1 was detected in immature DCs cultured according to Scheicher's but not to Inaba's protocol. In conclusion, Notch signalling resulting in induction of Hes1, Hes5 or Deltex1 was not observed.

Notch signalling has been studied in haematopoiesis. Notch activation is important in the differentiation of haematopoietic precursor, even though the data is very controversial: Notch may inhibit differentiation of myeloid precursors (Bigas *et al.*, 1998; Han *et al.*, 2000; Milner *et al.*, 1996) rather than promote differentiation (Schroeder and Just, 2000b). Activated Notch1 inhibited proliferation of progenitors (Schroeder and Just, 2000a; Walker *et al.*, 1999), whereas retroviral transduction of murine bone marrow cells with the intracellular domain of Notch1 induced immortalised mouse progenitor cell lines (Varnum-Finney *et al.*, 2000).

Less is known of the maturation of already differentiated myeloid cells such as monocytes to macrophages or iDCs to mature DCs. Monocytes cultured with immobilised Delta1 undergo apoptosis in the presence of M-CSF (Ohishi *et al.*, 2000), whereas they differentiate into DCs in the presence of GM-CSF (Mizutani *et al.*, 2000; Ohishi *et al.*, 2001). Cheng *et al.* found that Notch1 is important for maturation of DCs (Cheng *et al.*, 2001). They propose a role for Notch1 in maintaining the expression levels of several NF-κB subunits in DCs. In the absence of Notch1, TNFα-induced maturation of DCs is decreased because of the reduced level of NF-κB. However, TNFα-mediated maturation of DCs did not alter in the presence of immobilised Delta1 suggesting that activation of Notch by Delta1 did not affect the maturation process (Ohishi *et al.*, 2001). Furthermore, Radtke and colleagues have shown that Notch1 is not required at all for the development of DCs *in vivo* (Radtke *et al.*, 2000). In summary, the role of Notch signalling in DC differentiation and maturation remains unclear. My results suggest that Notch signalling mediated by induction of the Hes genes did not occur during the maturation process due to their low levels in immature and mature DCs.

In conclusion, Notch signalling did not seem to play a major role during maturation of DCs. However, the Notch ligands Jagged1 and Delta1 were differentially expressed during the maturation process. Expression pattern of Delta1 remains unclear and will need further investigations. TNFα-induced maturation of DCs upregulated Jagged1 more strongly in the presence than in the absence of IL-10. However, it is unlikely that Jagged1 plays a major role in the induction of anergic T cells by IL-10 modulated DCs because the same level of Jagged1 expression existed in LPS-matured DCs. Therefore, it is possible that the role of Jagged1 during the induction of an immune response is of a costimulatory rather than a modulating nature. Furthermore, posttranscriptional regulation of these molecules cannot be excluded.

So far, the expression but not the biological function of these molecules has been studied. It is now important to investigate whether expression of Notch ligands on APCs influences the outcome of an immune response.

V Analysis of T cell activation by L cells expressing Jagged1 or Delta1

V.1 Introduction

In the previous chapters I have shown that DCs upregulate Jagged1 and differentially express Delta1 upon maturation, whereas they constitutively express Notch1 and Notch2. Downstream components of the Notch signalling pathway were either not detected or not differentially regulated during DC maturation. In contrast, peripheral $CD4^+$ and $CD8^+$ T cells expressing Notch receptors 1-4 displayed differential regulation of downstream components of the Notch signalling pathway, such as Deltex and the Hes genes, upon stimulation. By differentially expressing Notch ligands or downstream components of the Notch pathway, respectively, a role for DCs as the "signalling cell" and T lymphocytes as the "receiving cells" could be envisaged.

To test this hypothesis, I used murine MHC class II-negative fibroblasts (L cells). They are derived from an $H\text{-}2^k$ background and in addition to the class I molecules $H\text{-}2K^k$, $H\text{-}2D^k$ and $H\text{-}2L^k$, they express endogenous B7.1. These cells were co-transfected with the class II molecule $I\text{-}A^b$ and either human Jagged1 or murine Delta1 and generously provided by K. Wong (Dept. Biology, Imperial College). Human Jagged1 shares an overall amino acid identity with murine Jagged1 of 96% (Shimizu *et al.*, 1999) and is capable of activating murine Notch receptors (K. Tan, unpublished data; Hoyne *et al.*, 2000). Therefore, human Jagged1 was used for the transfection of the L cells, since the murine counterpart was not yet cloned at that time. $I\text{-}A^b$ transfected L cells expressing Jagged1 or Delta1 are referred to as $Jagged1^+$ and $Delta1^+$ L cells, respectively.

The advantage of using this artificial APC cell line is that they can be transfected with individual components such as MHC class II molecules and Notch ligands. The transfected L cells express MHC/peptide complexes and costimulatory molecule B7.1, which together deliver "signal 1" and "signal 2" required for T cell activation. This creates conditions similar to the previous *in vitro* activation of $CD4^+$ and $CD8^+$ T lymphocytes by anti-CD3/anti-CD28,

but additionally allows Notch activation in T cells by using the L cells co-transfected with Notch ligands.

Here I have used the L cell transfectants in mixed lymphocyte reactions (MLRs). The advantage of a MLR is the high frequency of alloreactive T cells; it has been estimated that 1-5% of all T cells are alloreactive, which is far higher than the frequency of T cells reactive with a particular foreign antigenic peptide plus self-MHC molecule. An explanation for the high frequency of alloreactive T cells is that a particular TCR is not only specific for a foreign antigenic peptide plus self-MHC molecule but additionally can crossreact with certain allogeneic MHC molecules (Obst *et al.*, 1998; Smith *et al.*, 1997). Since allogeneic cells express on the order of 10^5 MHC class I molecules per cell, $CD8^+$ T cells bearing low-affinity cross-reactive receptors might be able to bind by virtue of the high density of alloantigen. Foreign antigen, on the other hand, is sparsely displayed on the membrane of an APC, limiting responsiveness to only those T cells bearing high-affinity receptors. Therefore, transfected L cells expressing high levels of MHC class I of the $H\text{-}2^k$ haplotype and the class II molecules $I\text{-}A^b$ should be capable of activating alloreactive $CD4^+$ and $CD8^+$ T lymphocyte derived from another background such as, for example, $H\text{-}2^d$.

V.2 Phenotyping of I-A^{b+} L cells co-transfected with Jagged1 or Delta1

Phenotyping of the transfected L cells by flow cytometry.

A bicistronic transcription system consisting of an internal ribosome entry site (IRES) and enhanced green fluorescent protein (EGFP) has been used to co-express the target gene (Jagged1 or Delta1) together with the reporter gene EGFP (see chapter VI for more details about EGFP; Crameri *et al.*, 1996; Rees *et al.*, 1996). This allowed identification of the co-transfected L cells by flow cytometry. After several rounds of sorting for EGFP expressing cells, an over 95% pure population of $EGFP^+$ L cells was obtained (Fig. V.1A). Because the gene for EGFP was set downstream of the gene of interest (Jagged1 or Delta1), Jagged1 or Delta1 have to be translated first in order to allow the translation of EGFP. Therefore, it was possible to determine indirectly the expression of Delta1 or Jagged1 by measuring the expression of EGFP. Jagged1 co-transfected I-A^{b+} L cells exhibited consistently a somewhat higher level of transgene expression than the $Delta1^+$ co-transfected I-A^{b+} L cells. Even though EGFP expression in the co-transfected cells seemed relatively low, it was an approximately ten-fold increase compared to the I-A^{b+} L cells.

The expression level of cell surface molecules involved in T cell activation was measured using flow cytometry (Fig. V.1B-D). Jagged1 and Delta1 co-transfected cells expressed the same amount of transfected MHC class II molecule I-A^b whereas the I-A^{b+} L cells showed slightly reduced I-A^b expression. The level of H-$2K^k$ expression differed slightly between the three cell types with the $Delta1^+$ L cells expressing most and the $Jagged1^+$ L cells expressing least. All three L cell types expressed same levels of B7.1.

The initiation of an immune response requires not only activation through the TCR and the CD28 molecule on the T cells but also antigen-independent interactions between adhesion molecules. For example ICAM-1 has been reported to function both as costimulator and as adhesion molecule enhancing the avidity of interactions between T cells and APC (Damle *et al.*, 1992; Okazaki *et al.*, 1994; Zuckerman *et al.*, 1998). ICAM1 was not detected on the L cells by flow cytometry (data not shown). However, it has been reported that ICAM1 expression by the APC is only essential in the case of low MHC class II expression and in the absence of any other adhesion molecules (Altmann *et al.*, 1989).

Other costimulatory molecules such as OX40L are predominantly found on professional APCs and have not been described on fibroblasts (Lane, 2000). CD40 expression is more promiscuous. Although its expression is mainly confined to subsets of leukocytes, endothelium and some epithelial cell lines (reviewed in Grewal and Flavell, 1998), CD40 has also been reported to be upregulated in fibroblasts upon IFNγ exposure (Fries *et al.*, 1995; Yellin *et al.*, 1995). However, CD40 expression on L cells has not been described yet.

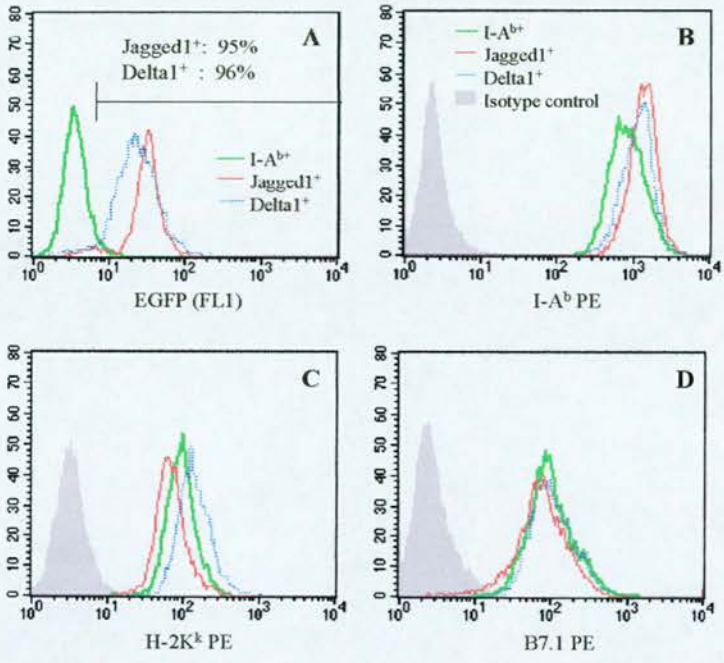


Figure V.1. Phenotyping of the transfected L cells by flow cytometry.
The L cells were detached using trypsin/EDTA.
[A] EGFP expression was detected in unstained cells by the FL1 detector. Percentages of EGFP$^+$ cells transfected with either Jagged1 or Delta1 are indicated in the within the histogram.
[B] Cells were stained for the transfected MHC class II molecule I-A^b.
[C] Cells were stained for the endogenous MHC class I molecule H-2K^k.
[D] Cells were stained for the costimulatory molecule B7.1.
Isotype control for one of the transfected L cells is shown as a representative. It did not differ between the three transfected L cell lines. Details of antibodies are given in table II.4 & II.5.

Gene expression of Notch pathway components in transfected L cells measured by real-time PCR.

To phenotype the L cells for gene expression, total RNA was extracted and checked for genomic DNA contamination as described in section II.2.15. Quality of RNA was ensured by running a RNA gel (Fig. V.2A). Gene expression of Notch receptors and ligands were analysed by RT-PCR (Fig. V.2B). As expected, only Jagged1$^+$ L cells expressed human Jagged1. Weak expression of endogenous Jagged1 in I-A^{b+} and Delta1$^+$ L cells was detected with the primers for murine Jagged1. In Jagged1$^+$ L cells, the primers for murine Jagged1 amplified both, endogenous and transgene transcripts for Jagged1. This is due to the crossreactivity of the primers with human Jagged1. L cells also expressed low amount of endogenous Delta1. However, compared to the high expression in Delta1$^+$ L cells, the endogenous Delta1 expression in Jagged1$^+$ and I-A^{b+} L cells is negligible. Differences in other components of the Notch pathway such as Notch1, 2 and 4, Jagged2 and Hes1 were not detected by RT-PCR which may be due to lack of sensitivity of this method. Therefore, components of the Notch pathway were additionally analysed by real-time PCR (Fig. V.3). Delta1 transfected L cells showed an over 3000-fold increase in Delta1 expression compared to Jagged1$^+$ or I-A^{b+} L cells. Comparing the increase of human Jagged1 in the Jagged1$^+$ L cells to the I-A^{b+} L cells was not possible due to the complete lack of human Jagged1 expression in I-A^{b+} L cells. Therefore, human Jagged1 expression was normalised to the universal calibrator described in method & material, section II.2.21. The Delta1$^+$ and I-A^{b+} L cells were negative for Deltex1 expression, again making normalisation impossible. Therefore, it only can be stated that Deltex1 is expressed in Jagged1$^+$ L cells, but not in Delta1$^+$ or I-A^{b+} L cells. The expression of endogenous Jagged2 and Delta3 was slightly increased in Jagged1$^+$ L cells, whereas Notch1, Notch3 and Hes1 did not exceed a 3-fold change in Jagged1$^+$ or Delta1$^+$ L cells compared to I-A^{b+} L cells. Therefore, the changes in their expression were not considered significant (method & material, II.2.21.4). Interestingly, Hes5 could not be detected in any of the L cell lines.

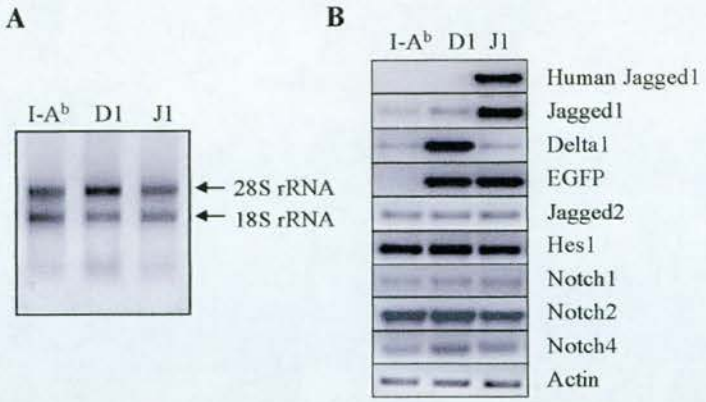


Figure V.2. Gene expression of Notch pathway components in transfected L cells
Transfected L cells were detached with Trypsin/EDTA and total RNA was extracted.
[A] RNA was tested by running a RNA gel as described in materials & methods, section II.2.15.
[B] The RNA was reverse transcribed and amplified by RT-PCR. The PCR-amplified products were run in a 2% agarose gel. β-Actin was used as a house keeping gene to normalise all the RNA samples to the same amount of starting template and as a control for the PCR reaction itself. I-A^b = I-A^{b+} L cells, J1 = Jagged1$^+$ L cells, D1 = Delta1$^+$ L cells

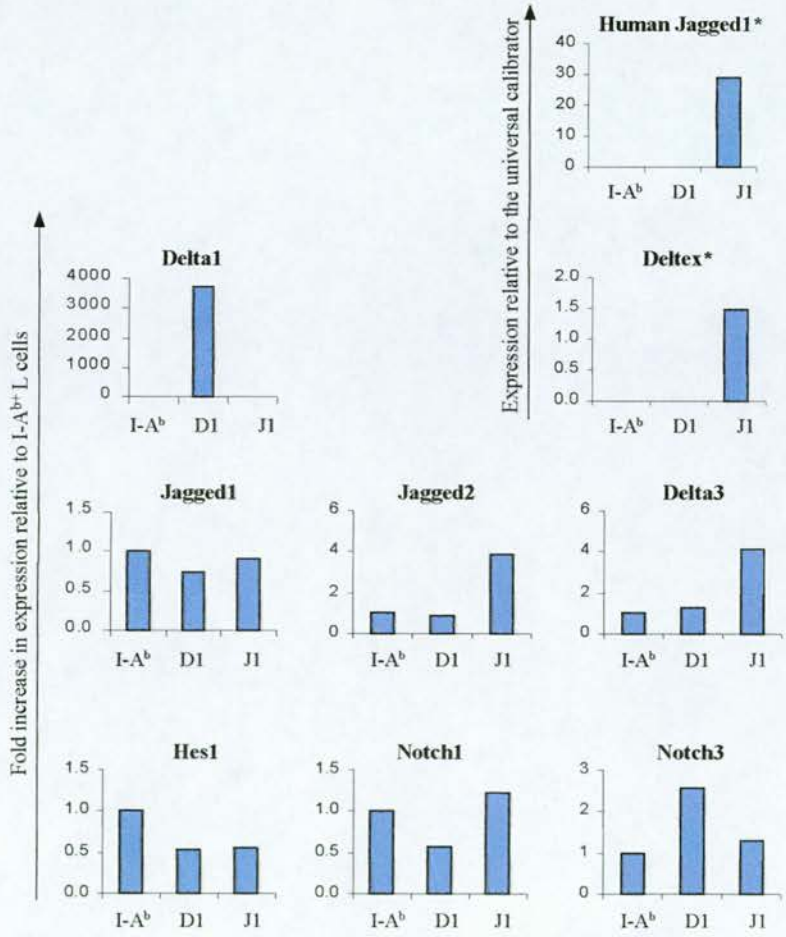


Figure V.3. Gene expression of Notch pathway components in transfected L cells measured by real-time PCR.
Transfected L cells were detached by trypsin/EDTA treatment. Total RNA was extracted and tested by running a RNA gel (Fig.V.2). RNA was reverse transcribed to cDNA and amplified by real-time PCR. Levels of mRNA transcripts are shown relative to the levels of the internal calibrator, which were I-A^{b+} L cells for all genes with exception of human Jagged1 and Deltex1. *)The calibrator for human Jagged1 and Deltex1 was the corresponding universal calibrator (see materials & methods, section II.2.21.) because these genes were not expressed in I-A^{b+} L cells. A change in expression of at least 3-fold or greater was considered significant. I-A^{b} = I-A^{b+} L cells, D1 = Delta1^{+} L cells, J1 = Jagged1^{+} L cells.

Trypsin does not cause a decrease in cell surface expression of B7.1, MHC class I or II molecules on the L cells.

L cells grow as an adherent monolayer. Trypsin in combination with the chelating agent EDTA was used to detach them. One concern is that the protease trypsin may partly digest cell surface proteins involved in T cell activation. To examine the effect of trypsin, the cells were either treated with EDTA alone for 20 minutes or with trypsin/EDTA for 5 minutes. Flow cytometry of B7.1, MHC class I and class II molecules showed that trypsin/EDTA did not cause a decrease in expression of these cell surface molecules (Fig. V.4). In contrast, EDTA produced a decreased expression of these molecules. It also was noted that EDTA-treated cells contained more dead cells than trypsin/EDTA-treated cells, which may be due to the prolonged treatment (20 vs. 5 minutes). Further, EDTA alone resulted in only partial disruption of the monolayer. Therefore, the combination of trypsin/EDTA was chosen for further experiments.

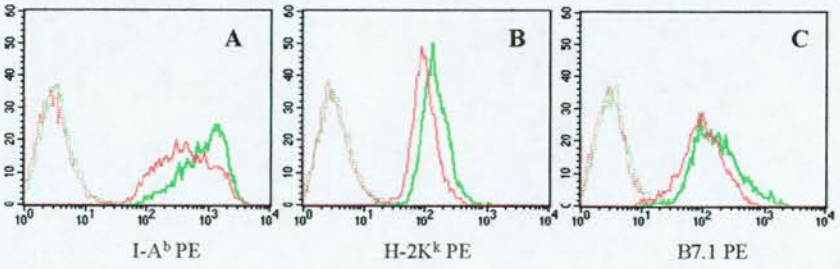


Figure V.4. Trypsin does not cause a decrease in cell surface expression of B7.1, MHC class I or II molecules on the L cells.
Transfected L cells were detached using either trypsin/EDTA or EDTA alone. PE-staining for I-A^{b} [A], H-2K^{k} [B], B7.1 [C] (solid lines) or the corresponding isotype controls (dashed lines) is shown for Delta1^{+} L cells. Green lines represent the trypsin/EDTA-detached L cells, the red lines the EDTA-detached L cells. Similar results were obtained for the other transfected cells (data not shown). Details of antibodies are given in table II.4 & II.5.

V.3 I-A^{b+} transfected L cells can induce a mixed lymphocyte reaction (MLR)

Since L cells are dividing rapidly, their proliferation has to be inhibited in order to measure T cell proliferation. Several methods exist to achieve this such as γ-irradiation, chemical fixation or DNA cross-linking reagents such as mitomycin C.

It is well known that higher doses of γ-irradiation are required to block cell division in transformed cell lines than in primary cells. However, Jagged1$^+$ L cells required an over twofold higher dose of γ-irradiation than I-A^{b+} L cells (data not shown). Therefore, irradiation was considered unsuitable as a blockage of cell division in this system. It has been reported that a 20-minute treatment with 1% paraformaldehyde inhibits the proliferation of L cells but not their capability to activate T cells in a MLR (Lechler *et al.*, 1985). Therefore, co-transfected and I-A^b transfected L cells were fixed with 1% paraformaldehyde. L cell proliferation was completely blocked after a 10-minute fixation. However, the fixed cells did not induce activation of T cells (data not shown). The same was found for a 10-minute fixation with 1% formaldehyde (data not shown). Following a method described by Altmann and colleagues (Altmann *et al.*, 1989), the cells were incubated with 100μg/ml mitomycin C for one hour. This treatment decreased proliferation substantially, but still allowed the L cells to activate T cells. This may be due to improved exposure of MHC molecules and/or the costimulatory molecule B7.1 on the surface of adherent L cells whereas complete inhibition of proliferation (and the resulting loss of adherence) rendered the L cells incapable of activating T cells.

$CD4^+$ T cells recognise MHC class II molecules on I-A^b transfected L cells.

$CD8^+$ T cells derived from the H-2^d haplotype should recognise MHC class I molecules of the H-2^k background, which are present on the L cells. Untransfected L cells, however, should not activate alloreactive $CD4^+$ T cells due to their lack of class II molecules. The specificity of alloreactive $CD4^+$ T cells for class II molecules was tested by comparing untransfected and I-A^b transfected L cells as stimulators in a MLR.

$CD4^+$ and $CD8^+$ T cells were purified by MACS from spleens of BALB/c mice ($H\text{-}2^d$ haplotype) and used as responder cells in a MLR with mitomycin C-treated untransfected or $I\text{-}A^b$ transfected L cells (Fig. V.5). Proliferation was measured on day 4. $CD8^+$ T cells proliferated strongly in response to both types of L cells whereas $CD4^+$ T cells proliferated only in response to $I\text{-}A^b$ expressing cells. This shows that transfection of L cells with an allogeneic MHC class II molecule is necessary and sufficient to activate alloreactive $CD4^+$ T cells.

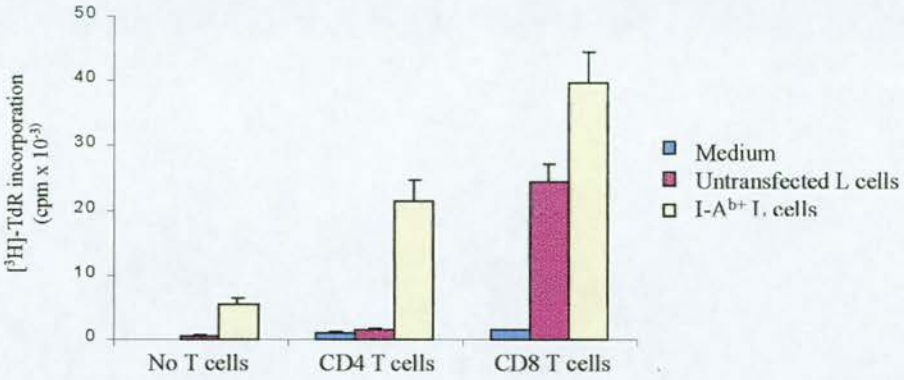


Figure V.5. $CD4^+$ T cells recognise MHC class II molecules on $I\text{-}A^b$ transfected L cells.
A MLR was carried out with $5x10^4$/well mitomycin C-treated untransfected or $I\text{-}A^b$ transfected L cells as stimulators and MACS-purified $CD4^+$ or $CD8^+$ T cells from BALB/c ($H\text{-}2^d$) spleens ($2x10^5$/well). After 4 days, proliferation was measured by $[^3H]$thymidine uptake during the last 18 hrs of culture and plotted as cpm x 10^{-3} ± standard deviation.

V.4 Jagged1 downregulates secretion of IFNγ by T cells in a MLR

To investigate the effect of Jagged1 and Delta1 on the induction of an immune response, Jagged1 or Delta1 co-transfected $I\text{-}A^{b+}$ L cells were used to activate alloreactive T cells in a MLR.

Jagged1 but not Delta1 expression in L cells downregulates release of IFNγ by $CD4^+$ T cells in a MLR.

MACS-purified $CD4^+$ T cells from BALB/c spleens were used in a MLR with mitomycin C-treated $Jagged1^+$, $Delta1^+$ and $I\text{-}A^{b+}$ L cells. Proliferation was measured on day 4 (Fig. V.6A). $Jagged1^+$, $Delta1^+$ and $I\text{-}A^{b+}$ L cells induced similar levels of $CD4^+$ T cell proliferation. Supernatants of the MLR cultures were tested for cytokine production after various time points. There were no detectable levels of IL-4 or IL-10 produced at any time point (data not shown). However, after 72 hours IFNγ was detected in the three cultures containing the different transfectants but at a lower level in the MLR induced by the $Jagged1^+$ L cells (Fig. V.6B). Even though the amount of proliferation and IFNγ secretion was variable between different experiments, the decrease of IFNγ secretion induced by $Jagged1^+$ L cells was reproducible.

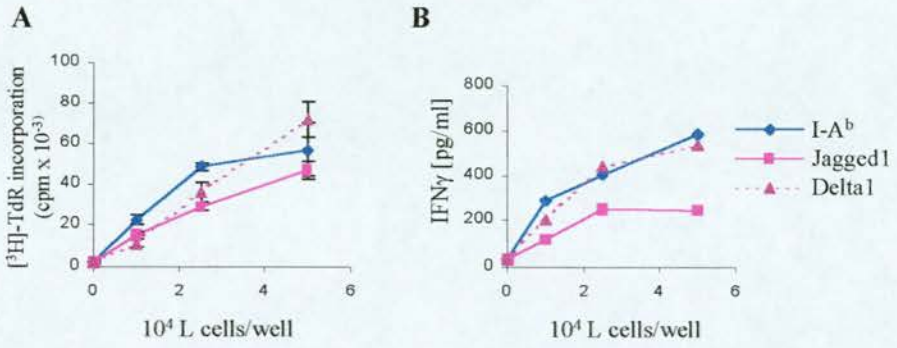


Figure V.6. Jagged1 but not Delta1 expression in L cells downregulates release of IFNγ by $CD4^+$ T cells in a MLR.

A MLR was carried out with varying numbers of mitomycin C-treated $Jagged1^+$, $Delta1^+$ or $I\text{-}A^{b+}$ L cells as stimulators and MACS-purified $CD4^+$ T cells from BALB/c spleens ($2x10^5$/well).

[A] After 4 days, proliferation was measured by $[^3H]$thymidine uptake during the last 18 hrs of culture and plotted as cpm x 10^{-3} ± standard deviation. The background proliferation of the L cells alone at their highest concentration did not exceed 4000 cpm (data not shown).

[B] After 72 hrs, the culture supernatants were harvested and tested for IFNγ by ELISA. $I\text{-}A^b$ = MLR with $I\text{-}A^{b+}$ L cells, Delta1 = MLR with $Delta1^+$ L cells, Jagged1 = MLR with $Jagged1^+$ L cells.

Jagged1 expression in L cells downregulates release of IFNγ by $CD8^+$ T cells in a MLR.

To investigate whether the reduction of IFNγ secretion by Jagged1 is $CD4^+$ T cell-specific or a general effect on T cells, the above mentioned experiment was repeated with $CD8^+$ T cells purified by MACS from spleens of BALB/c mice (Fig. V.7). Secretion of IFNγ was measured

after 48 hours because $CD8^+$ T cells are known to produce a high amount. Differences in proliferation and IFNγ secretion between different experiments were frequently observed. Therefore, two experiments are shown. In experiments #1 and #2, $Jagged1^+$ L cells had variable effects on proliferation compared to the $Delta1^+$ L cells but in both cases less IFNγ secretion was induced. The high variability between different experiments may be due to incomplete inhibition of L cell proliferation. However, reduced IFNγ secretion by $CD8^+$ T cells in response to $Jagged1^+$ L cells was observed consistently.

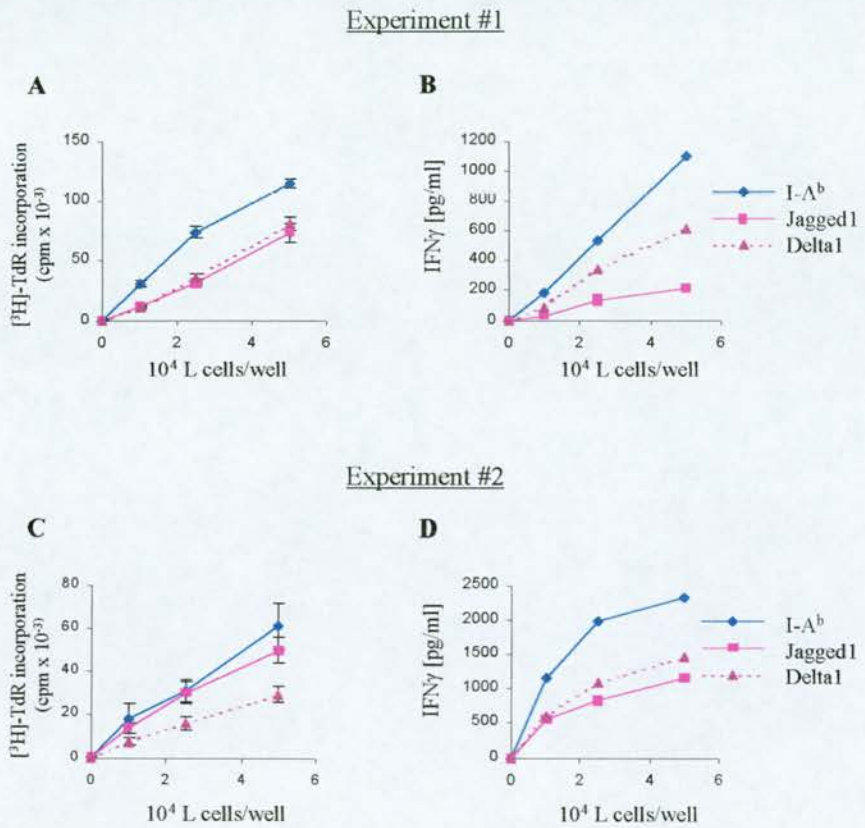


Figure V.7. Jagged1 expression in L cells downregulates release of IFNγ by $CD8^+$ T cells in a MLR.

A MLR was carried out with varying numbers of mitomycin C-treated $Jagged1^+$, $Delta1^+$ or $I\text{-}A^{b+}$ L cells as stimulators and MACS-purified CD8 T cells from BALB/c spleens ($2x10^5$/well). Two experiments are shown because of high variability between experiments.

[A] & [C], After 4 days, proliferation was measured by $[^3H]$thymidine uptake during the last 18 hrs of culture and plotted as cpm x 10^{-3} ± standard deviation. The background proliferation of the L cells at their highest concentration did not exceed 8000 or 4000 cpm in experiment #1 and #2, respectively.

[B] & [D], After 48 hrs, the culture supernatants were harvested and tested for IFNγ by ELISA.

$I\text{-}A^b$ = MLR with $I\text{-}A^{b+}$ L cells, Delta1 = MLR with $Delta1^+$ L cells, Jagged1 = MLR with $Jagged1^+$ L cells.

Jagged1 but not Delta1 expression in L cells downregulates release of IFNγ by nylon wool-enriched T cells in a MLR.

It is not entirely clear whether and to what extent purification by positive selection activates T cells. Therefore, an alternative method of T cell enrichment was used. Splenocytes were lympholyte-treated to deplete for dead cells and red blood cells and passed through a nylon wool column resulting in an unfractionated population consisting of 59±5% $CD4^+$ T cells and 20±5% $CD8^+$ T cells (material and methods, section II.3.6). Nylon wool-enriched T cells from BALB/c spleens were used in a MLR with mitomycin C-treated $Jagged1^+$, $Delta1^+$ or $I\text{-}A^{b+}$ L cells and proliferation was measured on day 4 (Fig. V.8). Proliferation of T cells in response to $Jagged1^+$ or $I\text{-}A^{b+}$ L cells did not differ significantly whereas T cells in response to $Delta1^+$ L cells proliferated more strongly compared to $I\text{-}A^{b+}$ L cells. Again, the level of IFNγ after 72 hours was greatly downregulated in the MLR with $Jagged1^+$ L cells. Small amounts of IL-2 were detected only after 72 hours and generally reflected the amount of proliferation.

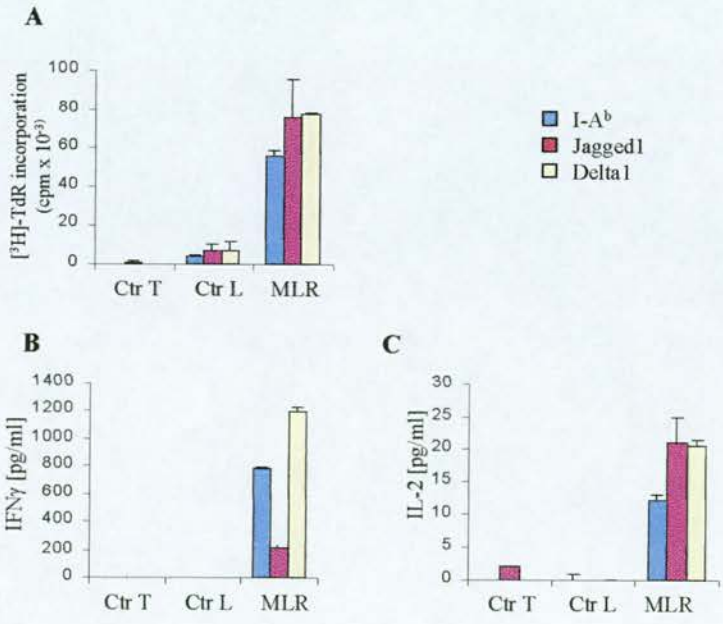


Figure V.8. Jagged1 but not Delta1 expression in L cells downregulates release of IFNγ by nylon wool-enriched T cells in a MLR.
A MLR was carried out with $2.5x10^4$ mitomycin C-treated Jagged1$^+$, Delta1$^+$ or I-A^{b+} L cells as stimulators and nylon wool-enriched T cells from BALB/c spleens ($2x10^5$/well).
[A] After 4 days, proliferation was measured by [^{3}H]thymidine uptake during the last 18 hrs of culture and plotted as cpm x 10^{-3} ± standard deviation.
[B] & [C] After 72 hrs, the culture supernatants were harvested and tested for IFNγ [B] and IL-2 [C] by ELISA.
I-A^b = MLR with I-A^{b+} L cells, Delta1 = MLR with Delta1$^+$ L cells, Jagged1 = MLR with Jagged1$^+$ L cells.

Due to the high variability in the baseline of proliferation and IFNγ production between different experiments, a statistical assessment of the IFNγ downregulation was required. Using the GraphPad InStat programme (GraphPad Software Inc., www.graphpad.com), the paired t test for 32 experiments led to following means, standard errors and two-tailed P values:

	L cells	Mean ± Standard Error
Proliferation: [cpm x 10^{-3}]	I-A^{b+} L cells	37.7 ± 3.8
	Jagged1$^+$ L cells	33.4 ± 4.0
	Delta1$^+$ L cells	46.6 ± 5.9
IFNγ secretion: [pg/ml]	I-A^{b+} L cells	1470 ± 530
	Jagged1$^+$ L cells	560 ± 300
	Delta1$^+$ L cells	1300 ± 580

Proliferation:	Jagged1 vs. I-A^b	P = 0.0577	Not significant
	Delta1 vs. I-A^b	P = 0.0254	Significant
IFNγ secretion:	Jagged1 vs. I-A^b	P = 0.0009	Very highly significant
	Delta1 vs. I-A^b	P = 0.0506	Not significant

In summary these results show that the Notch ligand Jagged1 expressed on I-A^{b+} L cells did not influence the proliferation of T cells whereas Delta1 expressed on I-A^{b+} L cells slightly increased T cell proliferation. However, Delta1 did not have an effect on IFNγ secretion, in contrast to Jagged1, which downregulated IFNγ secretion in T cells.

V.5 Decreased IFNγ secretion induced by Jagged1$^+$ L cells is not due to a soluble factor produced by the L cells

Downregulation of IFNγ is not due to a soluble factor produced by the Jagged1$^+$ L cells.

One possibility, which may account for the decreased amount of IFNγ induced by Jagged1$^+$ L cells, was the production of a soluble factor by the L cells. To investigate this possibility, L cell-conditioned media (LCCM) was titrated into the MLR.

L cell-conditioned media was obtained by growing L cells in complete RPMI for 48 hours before harvesting the media. Since the effect of Jagged1 on IFNγ induction was also observed in T cells enriched by nylon wool-purification, the following experiments were carried out with this population.

Nylon wool-enriched BALB/c T cells were stimulated with mitomycin C-treated I-A^{b+} L cells in the presence of different concentrations of L cell-conditioned media (Fig. V.9). MLRs with Jagged1$^+$ and Delta1$^+$ L cells were included as controls. No differences in proliferation or IFNγ secretion was observed by adding conditioned medium from either Jagged1$^+$, Delta1$^+$ or I-A^{b+} L cells to the MLR. However, proliferation and especially IFNγ secretion decreased with increasing amount of conditioned medium from all three of L cell transfectants. This was probably caused by depletion of nutrients in the conditioned medium.

Thus I have excluded the possibility that a soluble factor secreted by the L cells is causing the reduced IFNγ production by T cells stimulated with Jagged1$^+$ L cells.

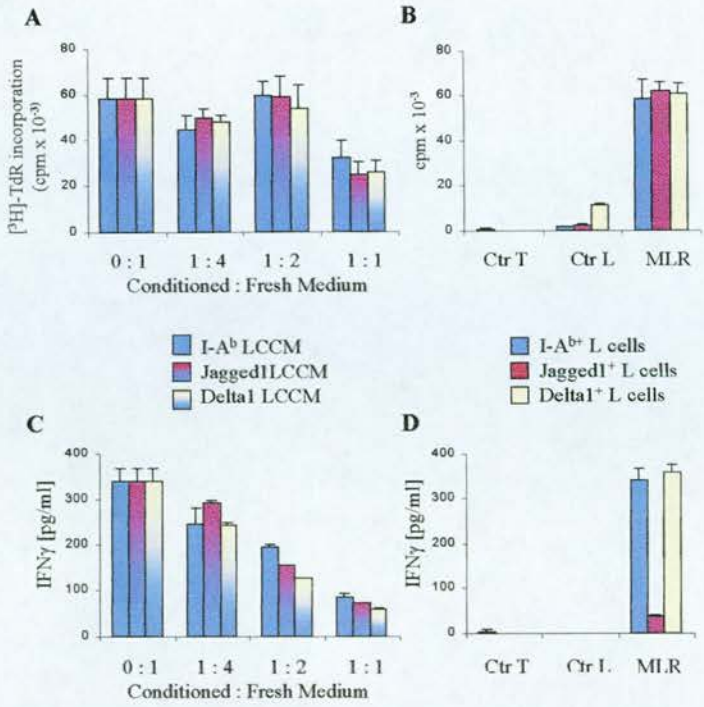


Figure V.9. Downregulation of IFNγ is not due to a soluble factor produced by the Jagged1$^+$ L cells.

L cell-conditioned medium from Jagged1$^+$, Delta1$^+$ or I-A^{b+} L cells (Jagged1, Delta1 and I-A^b LCCM, respectively) was titrated into a MLR with nylon-purified T cells ($2x10^5$/well) and mitomycin C-treated I-A^{b+} L cells ($2.5x10^4$/well) ([A] and [C]). As a control, a MLR with Jagged1$^+$, Delta1$^+$ and I-A^{b+} L cells was performed in parallel (Jagged1$^+$, Delta1$^+$ and I-A^{b+} L cells, respectively) ([B] and [D]).

[A] & [B], After 4 days, proliferation was measured by [^{3}H]thymidine uptake during the last 18 hrs of culture and plotted as cpm x 10^{-3} ± standard deviation.

[C] & [D], After 72 hrs, the culture supernatant of the titration experiment and of the control experiment were harvested and tested for IFNγ by ELISA.

V.6 L cells co-transfected with Jagged1 or Delta1 induce Notch signalling in T cells

The readout for Notch signalling on protein level is very difficult because antibodies cannot detect the activated intracellular portion of endogenous Notch. The most reliable measurement for Notch signalling at present is to evaluate transcription activation of downstream targets such as the Hes genes. It has been shown that Delta1 activates transcription of Hes1 through Notch signalling (Jarriault *et al.*, 1995; Jarriault *et al.*, 1998). Hes5 was reduced in Notch1

mutants and almost completely eliminated in CBF1 mutants, indicating that Hes5 is highly responsive to Notch signalling (de la Pompa *et al.*, 1997). Hes1 and Deltex1 are upregulated in transgenic thymocytes, which express constitutively active Notch1 and in T cell hybridomas retrovirally transfected with constitutively active Notch1 (Deftos *et al.*, 1998; Deftos *et al.*, 2000). Chang *et al.* also reported an increase in Hes1 transcripts in thymocytes TCRα-negative mice expressing constitutively active Notch1 (Chang *et al.*, 2000). Furthermore, monolayers of Jagged1-expressing HeLa cells induced transcription of Hes1 in cocultured Jurkat cells (Bash *et al.*, 1999). Therefore, upregulation of Hes transcripts was considered as a valid readout for Notch activation. Additionally, Deltex1 transcript may also be increased upon Notch activation even though direct evidence for this pathway is still missing.

Activating T cells in a MLR with Jagged1$^+$, Delta1$^+$ or I-A^{b+} L cells leads to increase of IL-2 transcripts and differential regulation of Notch signalling genes.

A MLR was carried out with nylon wool-enriched T cells from BALB/c spleens as responders and mitomycin C-treated L cells as stimulators. After 24, 48 or 72 hours, dead cells and cell debris were removed by lympholyte treatment and the cells prepared for FACS sorting by double staining with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 antibodies. Total RNA was extracted from the sorted CD4$^+$ and CD8$^+$ T cell populations and prepared for real-time PCR analysis (Fig. V.10A). The sample for CD8$^+$ T cells activated by Jagged1$^+$ L cells at 72 hrs was lost during RNA isolation. Expression of each gene is given relative to gene expression of the control CD4$^+$ T cells at 24 hours (Fig. V.10B). Gene expression in CD8$^+$ T cells are also shown relative to control CD4$^+$ T cells in order to compare relative amounts of transcript level between CD4$^+$ and CD8$^+$ T cells.

Hes1 expression was strongly upregulated at 48 hours in CD4$^+$ T cells and to a lesser extent in CD8$^+$ T cells isolated from the MLR stimulated with Jagged1$^+$ L cells. CD4$^+$ T cells sorted from the MLR with Delta1$^+$ L cells showed also an increase in Hes1 transcripts. On the other hand, there were no differences in Hes5 or Deltex1 transcripts. There was very little difference in expression of the Notch ligands or Notch receptors. IL-2 was induced in all activated CD4$^+$ T cells and to a much lesser extent in CD8$^+$ T cells at 24 hours. IFNγ was only very minimally induced in CD4$^+$ T cells. CD8$^+$ T cells sorted from the MLR with Delta1$^+$ or I-A^{b+} L cells produced a substantial amount of IFNγ whereas expression of IFNγ in CD8$^+$ T cells in response to Jagged1$^+$ L cells was reduced compared to the other two. IL-10 was not induced confirming data obtained by ELISA. IL-4 may have been induced in CD4$^+$ T cells by Delta1$^+$

L cells at 24 hours. Expression of IL-4 was not measured in $CD8^+$ T cells because IL-4 transcripts were not detected in previous experiments.

In conclusion, there is strong evidence of Notch signalling in $CD4^+$ T cells in response to $Jagged1^+$ and $Delta1^+$ L cells and in $CD8^+$ T cells in response to $Jagged1^+$ L cells because the Hes1 expression is increased 40- to 90-fold. IL-2 expression is induced to similar levels in response to all three types of L cell transfectants. IFNγ transcripts were only reduced by twofold in $CD8^+$ T cells in response to $Jagged1^+$ L cells compared with $CD8^+$ T cells in response to $I\text{-}A^{b+}$ or $Delta1^+$ L cells. This reduction did not exceed 50% and was even less in another experiment (data not shown).

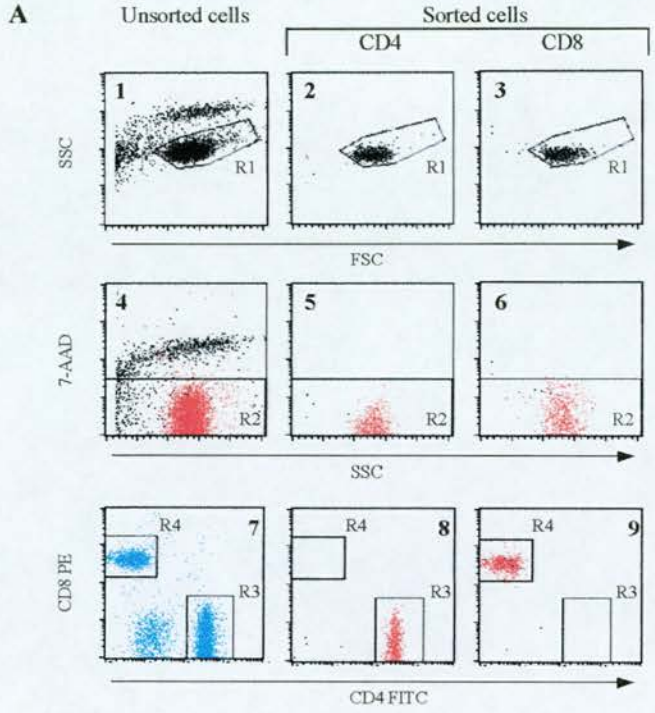
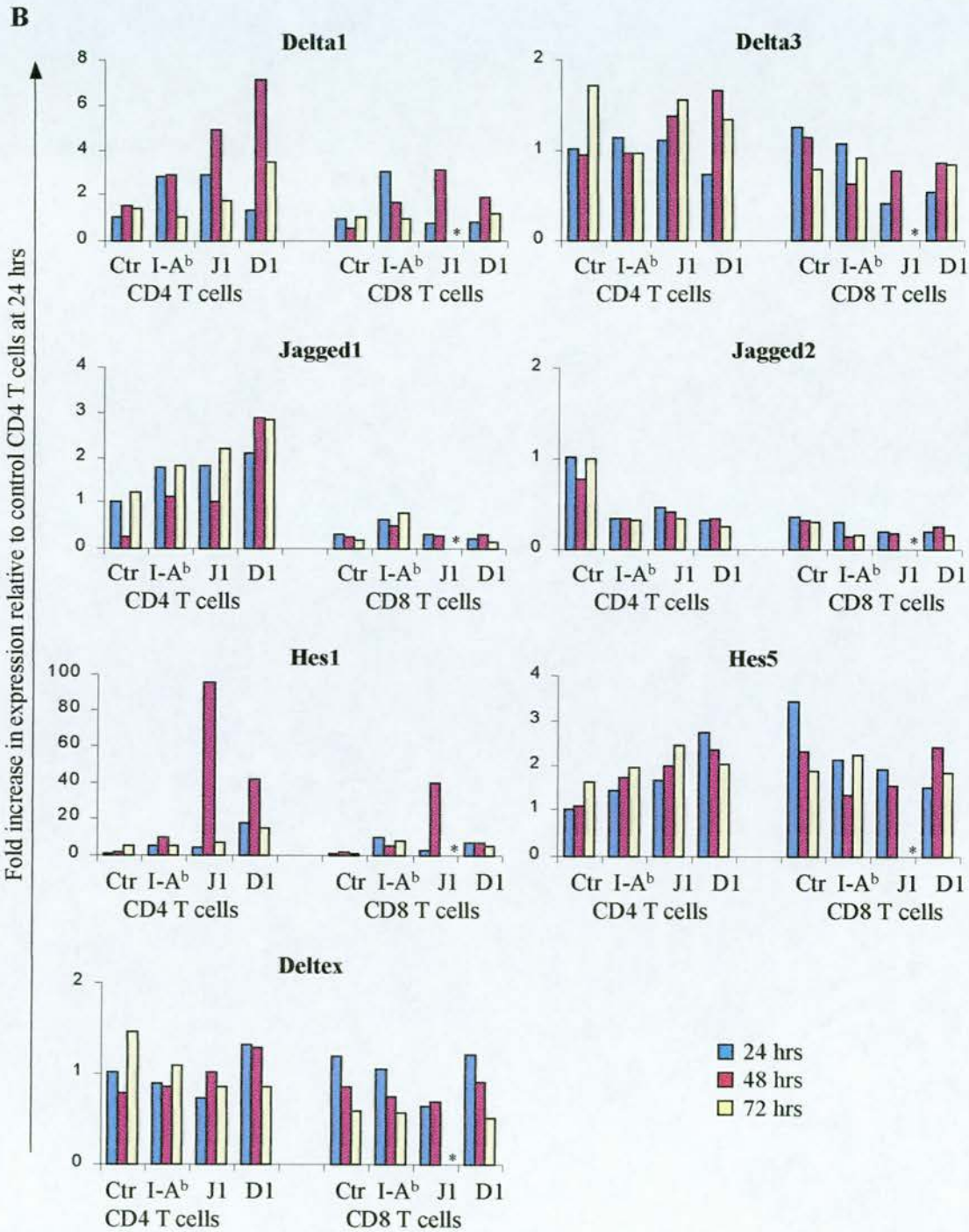
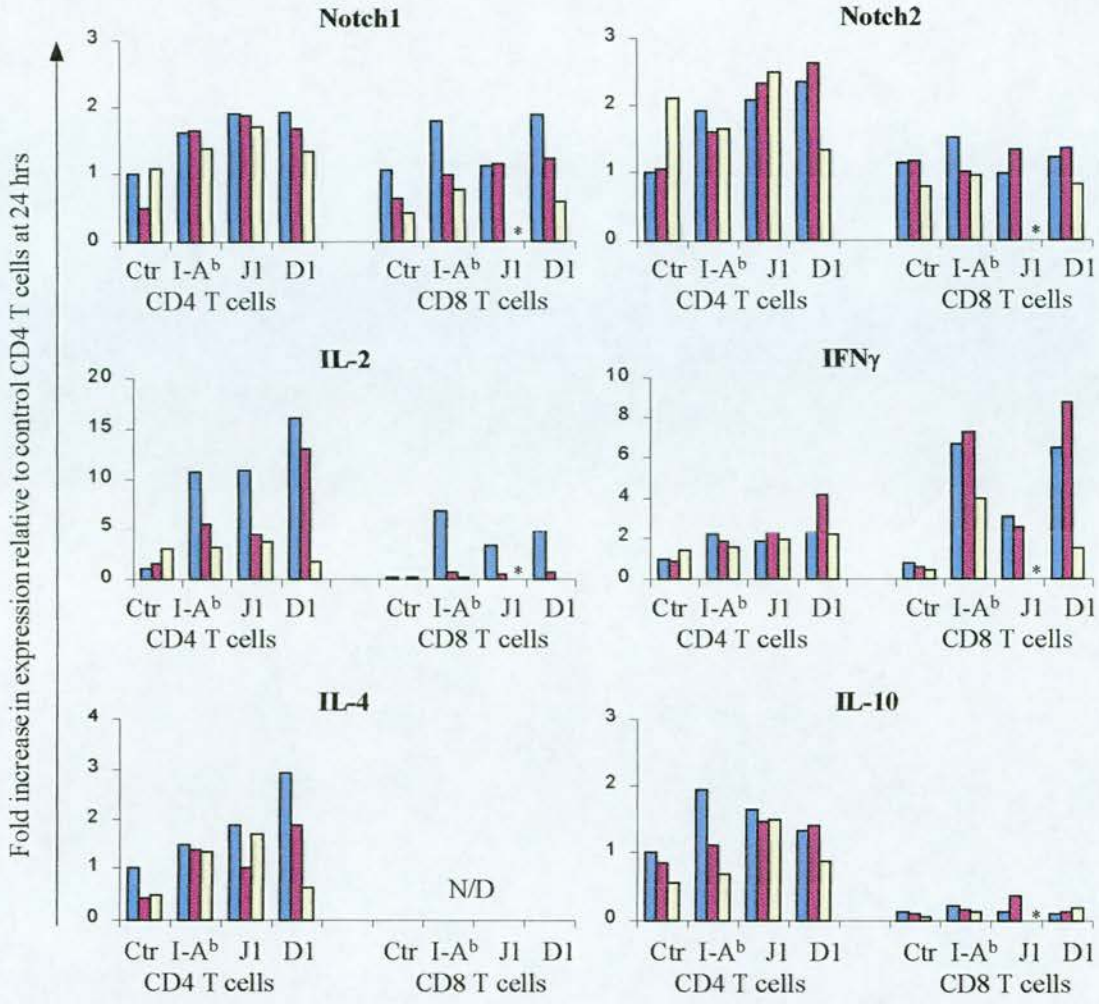


Figure V.10 A. Activating T cells in a MLR with $Jagged1^+$, $Delta1^+$ or $I\text{-}A^{b+}$ L cells leads to increase of IL-2 transcripts and differential regulation of Notch signalling genes.
A MLR was carried out with mitomycin C-treated $Jagged1^+$, $Delta1^+$ or $I\text{-}A^{b+}$ L cells as stimulators and nylon wool-enriched T cells from BALB/c spleens. Control T cells were incubated in medium.
[A] After 24, 48 or 72 hrs, cells were lympholyte-treated, double stained with FITC-CD4 and PE-CD8 and FACS sorted excluding dead cells and L cells by 7-AAD uptake. Dot plots #1-3 show forward scatter (FSC) and side scatter (SSC) analysis of ungated cells. Region R1 gated for lymphocytes. Dot plots #4-6 show ungated cells with R2 indicating viable cells. Cells falling into lymphogate R1 are represented by red dots. Dot plot #7 shows R1- and R2-gated cells (blue dots). Region R3 and R4 gated for $CD4^+$ and $CD8^+$ T cells, respectively. Dot plots #8 and #9 show $CD4^+$ and $CD8^+$ T cells, respectively, sorted by gating for lymphocytes (R1), viability (R2) and expression of CD4 or CD8 (R3 or R4, respectively). All cells are shown to include possible impurities. Sorted $CD4^+$ or $CD8^+$ T cells were over 98% pure. Details of antibodies are given in table II.4 & II.5.

[B] Sorted $CD4^+$ and $CD8^+$ T cells were processed for total RNA extraction and RNA was tested by RT-PCR as described in materials & methods, section II.2.16. RNA was reverse transcribed to cDNA and amplified by real-time PCR as described in materials & methods, section II.2.21. Levels of mRNA transcripts are shown relative to the levels of the internal calibrator, which were control $CD4^+$ T cells taken at 24 hrs. A change in expression of at least 3-fold or greater was considered significant. Ctr = Control T cells incubated in medium, I-A^b = MLR with I-A^{b+} L cells, J1 = MLR with $Jagged1^+$ L cells, D1 = MLR with $Delta1^+$ L cells.
*) Sample for $CD8^+$ T cells activated by $Jagged1^+$ L cells at 72 hrs was not determined.





V.7 Phenotyping of T cells activated in MLR with Jagged1$^+$ and Delta1$^+$ L cells by flow cytometry

The interaction of B7.1 with CTLA-4 downregulates responses to alloantigens in CD28-deficient mice and in the absence of CD28, the interaction of B7.1 but not B7.2 with CTLA-4 inhibits IFNγ production *in vitro* (Yamada *et al.*, 2001). Therefore, it is important to determine whether or not CTLA-4 is expressed on the cell surface of alloreactive T cells.

Only 7% of the T cells will be activated by alloantigens (Leiva *et al.*, 1997). To investigate the phenotype of the alloreactive T cells it is necessary to distinguish activated and resting T

cells. It is known that T cells upregulate selective cell surface molecules upon activation such as the IL-2 receptor (IL-2R) α chain, CD25. The IL-2R is a trimeric receptor consisting of the IL-2-specific α and β chains and the common γ chain (Minami and Taniguchi, 1995). It occurs in three forms that exhibit different affinities for IL-2: the low-affinity monomeric IL-2Rα, the intermediate-affinity dimeric IL-2Rβγ, and the high-affinity trimeric IL-2Rαβγ. Signal transduction by the IL-2R requires both the β and γ chains, but only the trimeric receptor containing the α chain as well binds IL-2 with high affinity. Because the α chain is expressed by activated but not resting T cells, it can be used to identify activated cells by flow cytometry.

Table V.1 summarises the average expression of CD25, the early activation marker CD69, CTLA-4 and the mean fluorescence of TCRβ for $CD4^+$ and $CD8^+$ T cells freshly isolated (*ex vivo*) from spleens. The values obtained for expression of CD25, CD69 and CTLA-4 broadly agree with published data (Craston *et al.*, 1997; Krummel and Allison, 1995; Sakaguchi *et al.*, 1995; Walunas *et al.*, 1994).

Table V.1. Phenotyping of *ex vivo* $CD4^+$ and $CD8^+$ T cells by flow cytometry.

	$CD4^+$ T cells	$CD8^+$ T cells
% $CD25^+$	6.6 ± 2.8	2.1 ± 1.2
% $CD69^+$	20.4 ± 8.8	14.3 ± 2.8
% $CTLA\text{-}4^+$	< 1.0	N/D
TCRβ*	760 ± 230	330 ± 60

N/D: not determined
* Data represent geometrical mean fluorescence

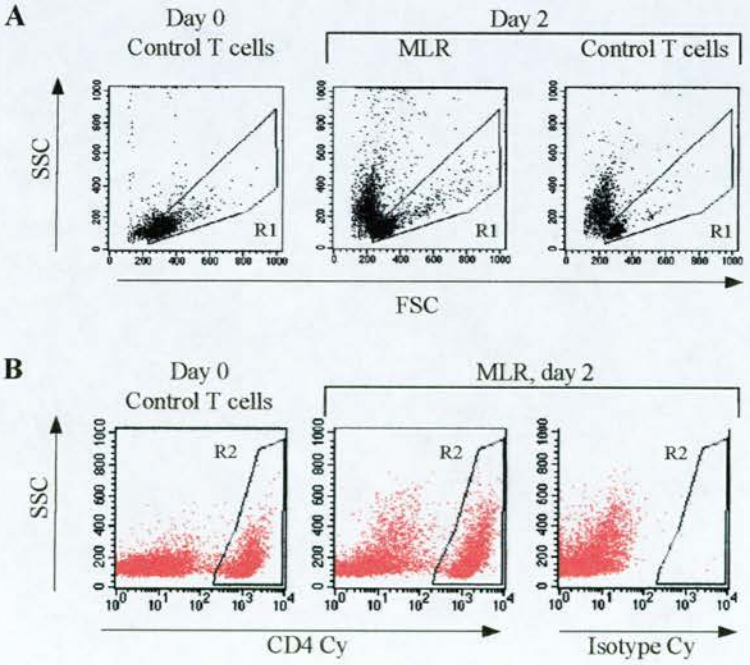
Phenotyping of $CD4^+$ T cells activated in MLR with $Jagged1^+$, $Delta1^+$ or $I\text{-}A^{b+}$ L cells by flow cytometry.

Nylon wool-enriched T cells were stimulated with mitomycin C-treated L cells for 2 days before phenotyping by flow cytometry (Fig. V.11). Freshly isolated cells (control T cells, day 0) and cells incubated for two days in medium (control T cells, day 2) were also analysed by flow cytometry to check for changes of phenotype due to culturing.

To distinguish alloreactive from resting T lymphocytes, cells were gated for CD4 staining and additionally stained for CD25 and the other molecules of interest. The quadrant grid to separate PE-positive and -negative cells had to be set at an angle due to incomplete compensation of the FITC-stained CD25 molecule. Over 99% of the CD4-gated cells expressed TCRβ, thus highly autofluorescent, $EGFP^+$ L cells were excluded by this gating

setting. Expression of the TCR, as measured by the mean fluorescence of TCRβ, increased on $CD4^+$ T cells cocultured with $Jagged1^+$ and with $Delta1^+$ L cells. However, this increase was non-specific as it was seen in CD25-positive and -negative $CD4^+$ T cells and could not be reproduced. Following coculture with the different L cells, the number of activated $CD4^+$ T cells increased approximately 2-3 fold. $CD4^+$ T cells activated by $Jagged1^+$ L cells, $I\text{-}A^{b+}$ L cells or $Delta1^+$ L cells increased from 3.8% to 13.3, 15.3 and 17.1%, respectively. These changes were reproduced. Over 50% of the CD25-positive population of $CD4^+$ T cells activated in the MLR expressed the early activation marker CD69. CTLA-4 was not detected on the cell surface of activated $CD4^+$ T cells. Number of $CD25^+$ and $CD69^+$ $CD4^+$ T cells decreased during the 2-day culture (control T cells, day 0 vs. day 2), which may be due to cell death or downregulation of the molecules. There was an increase of CTLA-4 expression on the control T cells incubated in medium for 2 days. Partly this may be due to non-specific staining which increases on dying cells.

These data show that the $CD4^+$ T cells activated by any of the three L cell transfectants did not differ in expression of the activation marker CD25, CD69 nor did they downregulate the TCR or upregulate CTLA-4. A limitation to this assay was, however, that $CD4^+$ T cells activated during the MLR could not be distinguished from effector $CD4^+$ T cells previously activated *in vivo* or from the population of regulatory $CD4^+$ T cells which constitutively express CD25 (Sakaguchi *et al.*, 1995).



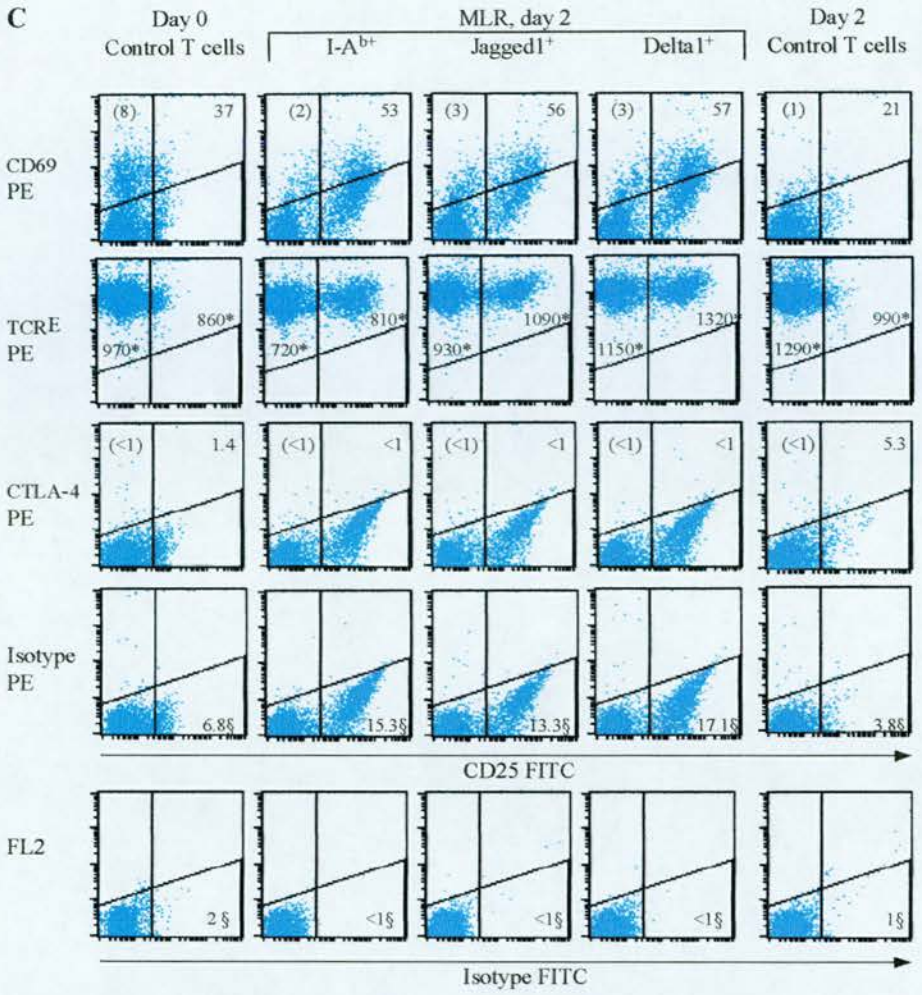


Figure V.11. Phenotyping of CD4^{+} T cells activated in MLR with Jagged1^{+}, Delta1^{+} or I-A^{b+} L cells by flow cytometry.

A MLR was carried out with mitomycin C-treated Jagged1^{+}, Delta1^{+} or I-A^{b+} L cells as stimulators and T cells which were enriched from BALB/c spleens by nylon wool purification. After 2 days, cells were triple stained for CD4 and CD25 and expression of CD69, TCRβ or CTLA-4 and analysed by flow cytometry. Control T cells were stained at day 0 or incubated in medium for 2 days.

[A] Forward scatter (FSC) and side scatter (SSC) analysis of control T cells at day 0 and day 2 and a representative MLR at day 2 are shown. Region R1 gated for live lymphocytes.

[B] FL3 (Cy) and side scatter (SSC) analysis of R1-gated cells of the T cell control at day 0 and a representative MLR at day 2 stained for CD4 or isotype control. Region R2 was used to gate for CD4^{+} T cells.

[C] Dot plot analysis with PE-conjugated antibodies (FL2) and FITC-conjugated anti-CD25 or isotype control (FL1) of gated CD4^{+} T cells of the MLRs (Jagged1^{+}, Delta1^{+} and I-A^{b+}). Quadrant grid was set using isotype staining.

Numbers within dot plots represent percentage of PE-stained cells of the CD25^{+} population and (percentage of PE-stained cells of the CD25^{-} population). §) Numbers represent percentage of FITC-stained cells. *) Numbers represent the geometrical mean fluorescence of TCRβ staining of the cell population in the corresponding quadrant.

Details of antibodies are given in table II.4 & II.5.

Phenotyping of proliferating T cells activated in MLR with Jagged1$^+$, Delta1$^+$ or I-A^{b+} L cells by flow cytometry.

To circumvent this limitation, CFSE (carboxyfluorescein diacetate succinimidyl ester) labelling was used to track the proliferating population of alloreactive T cells (materials & methods, II.3.7).

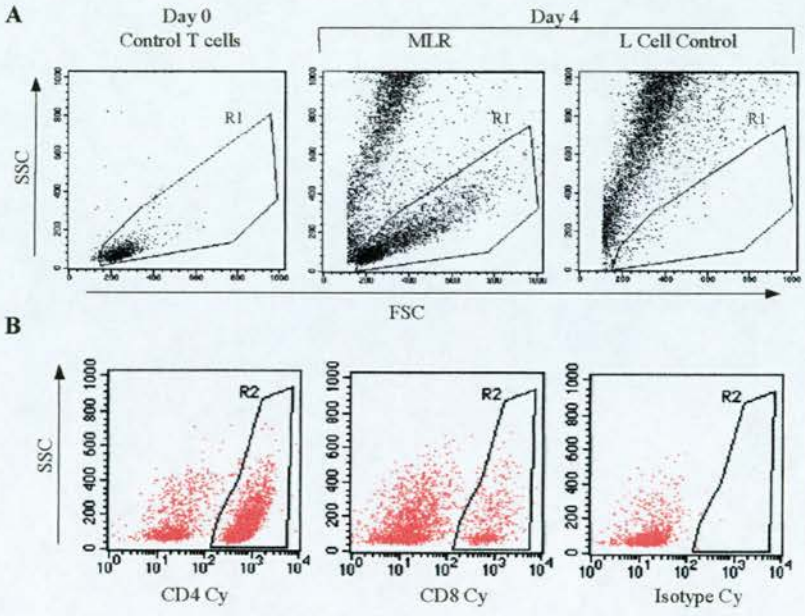
Nylon wool-enriched T cells were labelled with CFSE and used in a MLR with mitomycin C-treated L cells. After 4 days, cells were treated with lympholyte to remove dead cells and cell debris which can interfere with the flow cytometry analysis, resulting in increased background staining (Fig. V.11C, CTLA-4 staining of T cell control at day 2). Freshly isolated T cells were used as a control (control T cells, day 0). They were not CFSE-labelled because the cells would have been too bright to compensate for flow cytometry within the first 24 hours (materials & methods, II.3.7; Hasbold *et al.*, 1999). To ensure that the settings did not include highly autofluorescent, EGFP$^+$ L cells, the CD4- and the CD8-gated cells were stained for TCRβ expression (>99% in all samples) (Fig. V.12C & D). Quantitative comparison of cell division is difficult because dead cells have been removed altering the proportion of proliferating to resting cells. Further, a computer-based fitting programme would be required to calculate the cell number per division (Hasbold *et al.*, 1999). Therefore, percentages of stained vs. unstained cells were given separately for proliferating cells (CFSE^{low}) and for resting cells (CFSE^{high}). The quadrant grid for separation of proliferating and resting cells was set using CFSE-labelled cells incubated in medium for four days (data not shown).

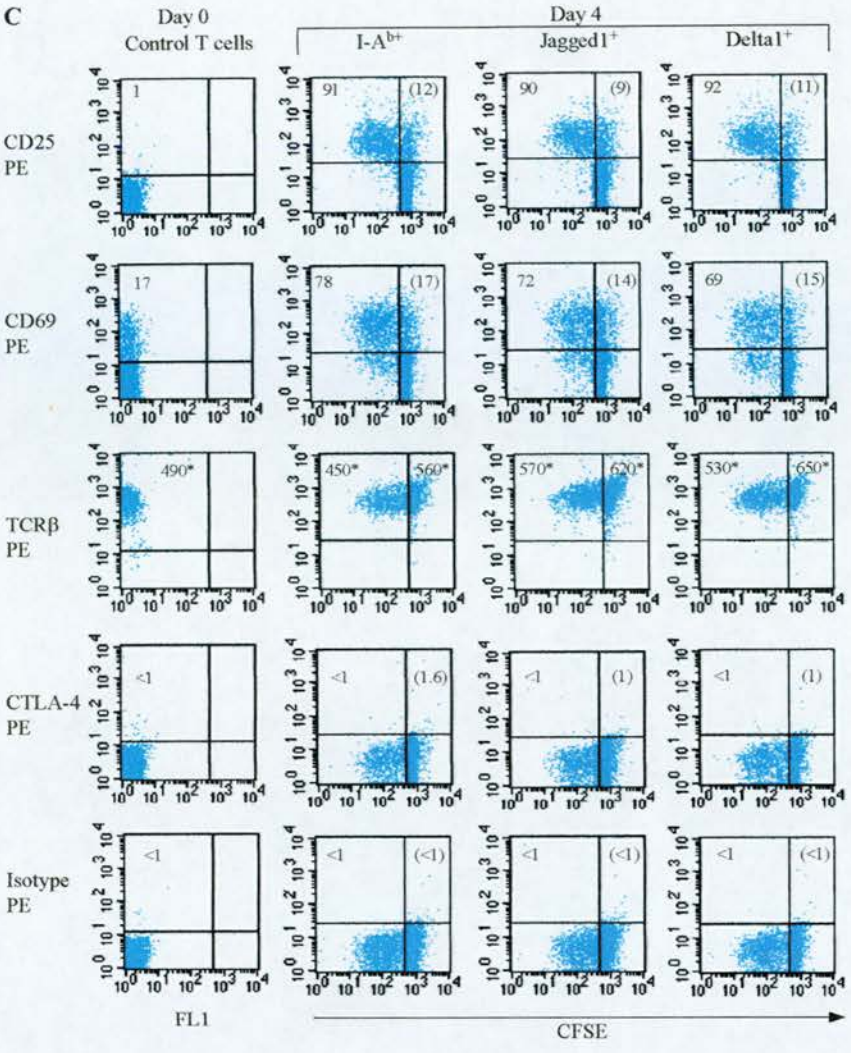
Culturing CD4$^+$ T cells seemed to induce a slight upregulation of the TCR comparing the mean fluorescence between freshly isolated CD4$^+$ T cells (control T cells, day 0) and the resting population in the MLRs (CFSE^{high}). Activation caused a small decrease of the TCR. However, Jagged1$^+$ L cells did not induce downregulation of the TCR in CD4$^+$ T cells. Over 90% of the proliferating CD4$^+$ T cells (CFSE^{low}) expressed CD25 and 70-80% CD69 (Fig. V.12C). 9-12% of the resting population (CFSE^{high}) showed CD25 expression whereas only 1% of the CD4$^+$ T cells *ex vivo* expressed CD25 (control T cells, day 0). Percentage of CD69 expressing cells did not alter between *ex vivo* CD4$^+$ T cells and resting CD4$^+$ T cells from the MLRs (14-17%). All CD4$^+$ T cells, however, were negative for staining of cell surface CTLA-4.

Proliferating CD8$^+$ T cells from the culture with Delta1$^+$ L cells expressed slightly more CD25 than CD8$^+$ T cells from cultures with Jagged1$^+$ or I-A^{b+} L cells (Fig. V.12D). 10-12% of the

resting $CD8^+$ T cells ($CFSE^{high}$) expressed CD25 whereas only 1% of the *ex vivo* $CD8^+$ T cells (control T cells, day 0) were CD25-positive. Over 90% of the proliferating $CD8^+$ T cells expressed CD69. Percentage of $CD69^+$ $CD8^+$ T cells did not change between the *ex vivo* $CD8^+$ T cells and the resting $CD8^+$ T cells ($CFSE^{high}$) from the MLR with $Jagged1^+$ or $I\text{-}A^{b+}$ L cells. A slightly higher percentage of the resting $CD8^+$ T cells from the MLR with $Delta1^+$ L cells expressed CD69. No changes of mean fluorescence of TCRβ were observed in any of the $CD8^+$ T cell populations.

Concluding from these data, proliferating cells had an activated phenotype ($CD25^+$ and $CD69^+$), but did not express CTLA-4 excluding its involvement in the observed decrease of IFNγ secretion induced by Jagged1. The TCR was not downregulated in $CD4^+$ or $CD8^+$ T cells in response to $Jagged1^+$ L cells excluding this as a explanation for decreased IFNγ secretion. Similar results were obtained at an earlier time point (day 3, data not shown).




C
Day 0
Control T cells
Day 4
I-A^{b+}
Jagged1^{+}
Delta1^{+}
CD25 PE
CD69 PE
TCRβ PE
CTLA-4 PE
Isotype PE
1
91
(12)
90
(9)
92
(11)
17
78
(17)
72
(14)
69
(15)
490*
450*
560*
570*
620*
530*
650*
<1
(1.6)
(1)
(<1)
FL1
CFSE

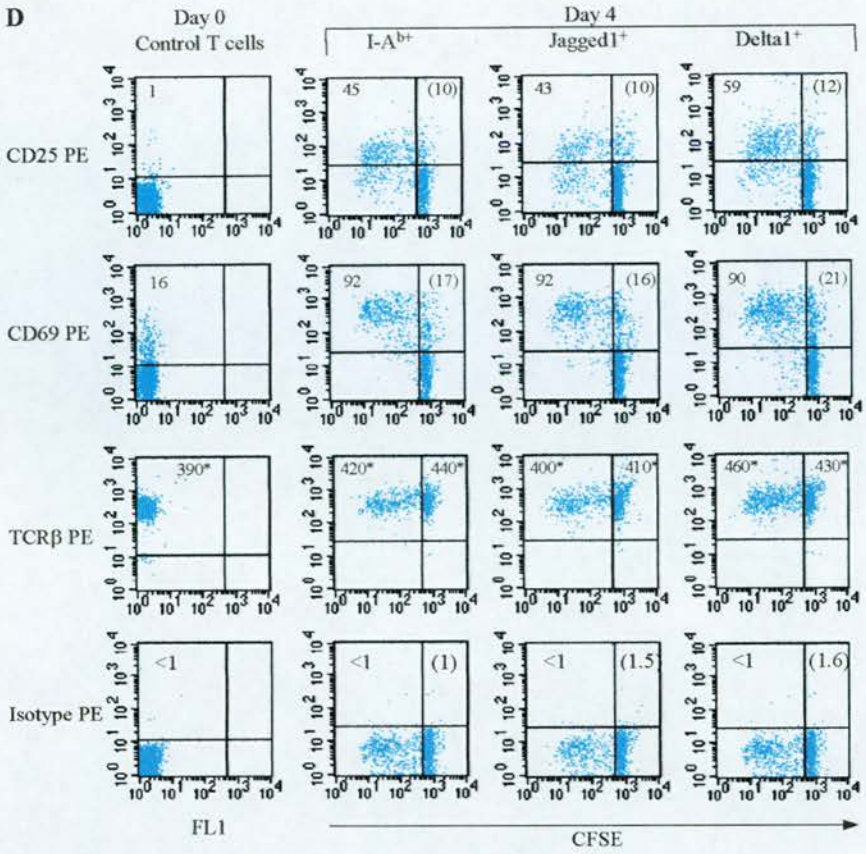


Figure V.12. Phenotyping of proliferating T cells activated in MLR with Jagged1^{+}, Delta1^{+} or I-A^{b+} L cells by flow cytometry.

A MLR was carried out with mitomycin C-treated Jagged1^{+}, Delta1^{+} or I-A^{b+} L cells as stimulators and CFSE-labelled T cells which were enriched from BALB/c spleens by nylon wool purification. Control L cells were incubated in medium (L cell control). After 4 days, cells were stained for CD4 or CD8 and expression of TCRβ, CTLA-4 and costimulatory molecules CD25 and CD69 and analysed by flow cytometry. CFSE-unlabelled control T cells were stained at day 0.

[A] Forward scatter (FSC) and side scatter (SSC) analysis with lymphocyte gate R1 of control T cells, a representative MLR and pooled Jagged1^{+}, Delta1^{+} and I-A^{b+} control L cells are shown.

[B] FL3 (Cy-Chrome) and side scatter (SSC) analysis of R1-gated cells of a representative MLR stained with anti-CD4, anti-CD8 or isotype control. Region R2 was used to gate for CD4^{+} or CD8^{+} T cells.

[C] & [D], Dot plot analysis with PE-conjugated antibodies (FL2) and CFSE (FL1) of gated CD4^{+} or CD8^{+} T cells ([C] and [D], respectively) of the MLRs (Jagged1^{+}, Delta1^{+} and I-A^{b+}). Quadrant grid was set using isotype staining. Numbers within the dot plots represent percentage of PE-stained cells of the proliferating population (CFSE^{low}) and (of the resting population (CFSE^{high})). *) Numbers represent the geometrical mean fluorescence of TCRβ staining of the cell population in the corresponding quadrant.

Details of antibodies are given in table II.4 & II.5.

V.8 Notch ligands expressed on L cells do not affect cell death of T cells during a MLR

Notch receptors and ligands have been shown to play a controversial role in cell survival. Notch1 provides protection to T cell lines from TCR-mediated apoptosis (Jehn *et al.*, 1999) whereas constitutively active Notch1 induces cell cycle arrest and apoptosis in B cells (Morimura *et al.*, 2000). Delta1 and Jagged2 promote proliferation and decrease apoptosis of haematopoietic progenitors (Han *et al.*, 2000; Tsai *et al.*, 2000). On the other hand, an immobilised truncated form of Delta-1 induces apoptosis in monocytes in the presence M-CSF, but not GM-CSF (Ohishi *et al.*, 2000). In conclusion, Notch signalling can induce or inhibit apoptosis depending on the cell type, the presence of cytokines and the experimental system used. In my system, I have investigated induction of T cell apoptosis upon activation by Jagged1 expressing L cells as a potential mechanism to explain the decrease in IFNγ.

Downregulation of IFNγ produced by T cells in MLR with Jagged1$^+$ L cells is not due to increased cell death.

T cells were cultured with L cells as described above. After 5 days, the CD4- or CD8-gated cells were stained with Annexin V and 7-AAD (Fig. V.13). Annexin V detects early and late apoptotic and necrotic cells whereas 7-AAD only stains late apoptotic and necrotic cells (Vermes *et al.*, 1995). Therefore, cells showing Annexin V and 7-AAD staining were referred to as dead cells since distinction between apoptosis and necrosis is not possible. There were only marginal differences in the numbers of dead CD4$^+$ T cells between the different MLRs. In CD8$^+$ T cells Delta1 seemed to induce whereas Jagged1 inhibited cell death compared to CD8$^+$ T cells from the MLR with I-A^{b+} L cells. Again, one has to bear in mind that a distinction between activated and resting T cells could not be made. Therefore, it is unclear whether differences in cell death of alloreactive T cells were masked by cell death of resting T cells.

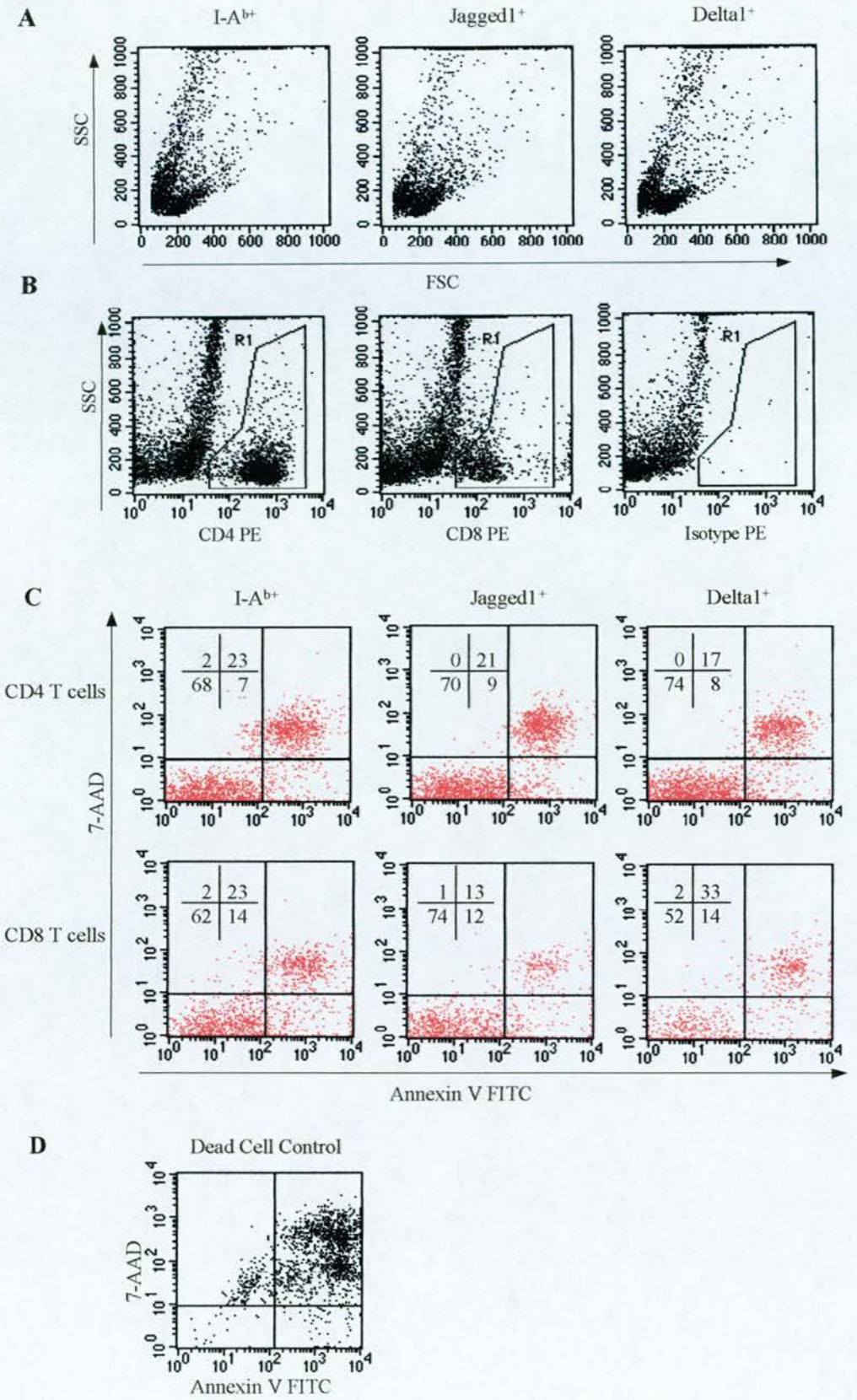

A
I-Ab+
Jagged1+
Delta1+
SSC
FSC
B
R1
CD4 PE
CD8 PE
Isotype PE
C
CD4 T cells
CD8 T cells
7-AAD
Annexin V FITC
2 23 68 7
0 21 70 9
0 17 74 8
2 23 62 14
1 13 74 12
2 33 52 14
D
Dead Cell Control
7-AAD
Annexin V FITC

Figure V.13. Downregulation of IFNγ produced by T cells in MLR with Jagged1$^+$ L cells is not due to increased cell death.

A MLR was carried out with mitomycin C-treated Jagged1$^+$, Delta1$^+$ or I-A^{b+} L cells as stimulators and nylon wool-enriched T cells from BALB/c spleens. After 5 days, cells were stained for CD4 or CD8, Annexin V and the viability dye 7-AAD was added prior to flow cytometry analysis as described in section II.3.9. As a positive control for Annexin V and 7-AAD staining, cells pooled from the MLR with Jagged1$^+$, Delta1$^+$ and I-A^{b+} L cells were incubated at 95°C for 2 mins (dead cell control).

[A] Forward scatter (FSC) and side scatter (SSC) analysis of the MLRs with Jagged1$^+$, Delta1$^+$ and I-A^{b+} L cells (Jagged1$^+$, Delta1$^+$ and I-A^{b+}, respectively) are shown.

[B] FL2 (PE) and side scatter (SSC) analysis of cells of a representative MLR stained for CD4, CD8 or isotype control. Region R1 was used to gate for CD4$^+$ or CD8$^+$ T cells.

[C] Dot plot analysis with FITC-conjugated Annexin V (FL1) and 7-AAD (FL3) of gated CD4$^+$ or CD8$^+$ T cells (red dots) of the MLRs (Jagged1$^+$, Delta1$^+$ and I-A^{b+}) and of ungated dead cell control ([D]).

Numbers in the cross within dot plots represent percentages within the corresponding quadrant. Details of antibodies are given in table II.4 & II.5.

Downregulation of IFNγ produced by T cells in MLR with Jagged1$^+$ L cells is not due to increased cell death of proliferating T cells.

To distinguish proliferating and resting cells, T cells were CFSE-labelled prior to adding to the L cells. After 5 days, cells were stained for Annexin V and CD4 or CD8 and analysed by flow cytometry (Fig. V.14). Percentages of Annexin V-positive cells were given for resting and proliferating cells and additionally for the entire population since no cells were removed by lympholyte treatment in contrast to the previous experiment with CFSE (Fig. V.12).

In the MLR with I-A^{b+} L cells a slightly smaller number of proliferating CD4$^+$ T cells were Annexin V-positive compared to the MLRs with Jagged1$^+$ and Delta1$^+$ L cells. There was no difference in Annexin V-positive CD8$^+$ T cells between the MLR with Jagged1$^+$ or I-A^{b+} L cells. CD8$^+$ T cells cultured with Delta1$^+$ L cells were less Annexin V-positive.

Small differences in T cell death seemed to exist between the different MLRs. Delta1 protected proliferating CD8$^+$ T cells from apoptosis, whereas both, Jagged1 and Delta1 slightly promoted apoptosis of alloreactive CD4$^+$ T cells. The same applied to the entire CD4$^+$ or CD8$^+$ T cell population and therefore, differed from the results obtained in the previous experiment (Fig. V.13). In conclusion, the observed differences in cell death were small, not consistent and hence regarded as insignificant. Additionally, trypan blue exclusion assays did not give reproducible differences (data not shown).

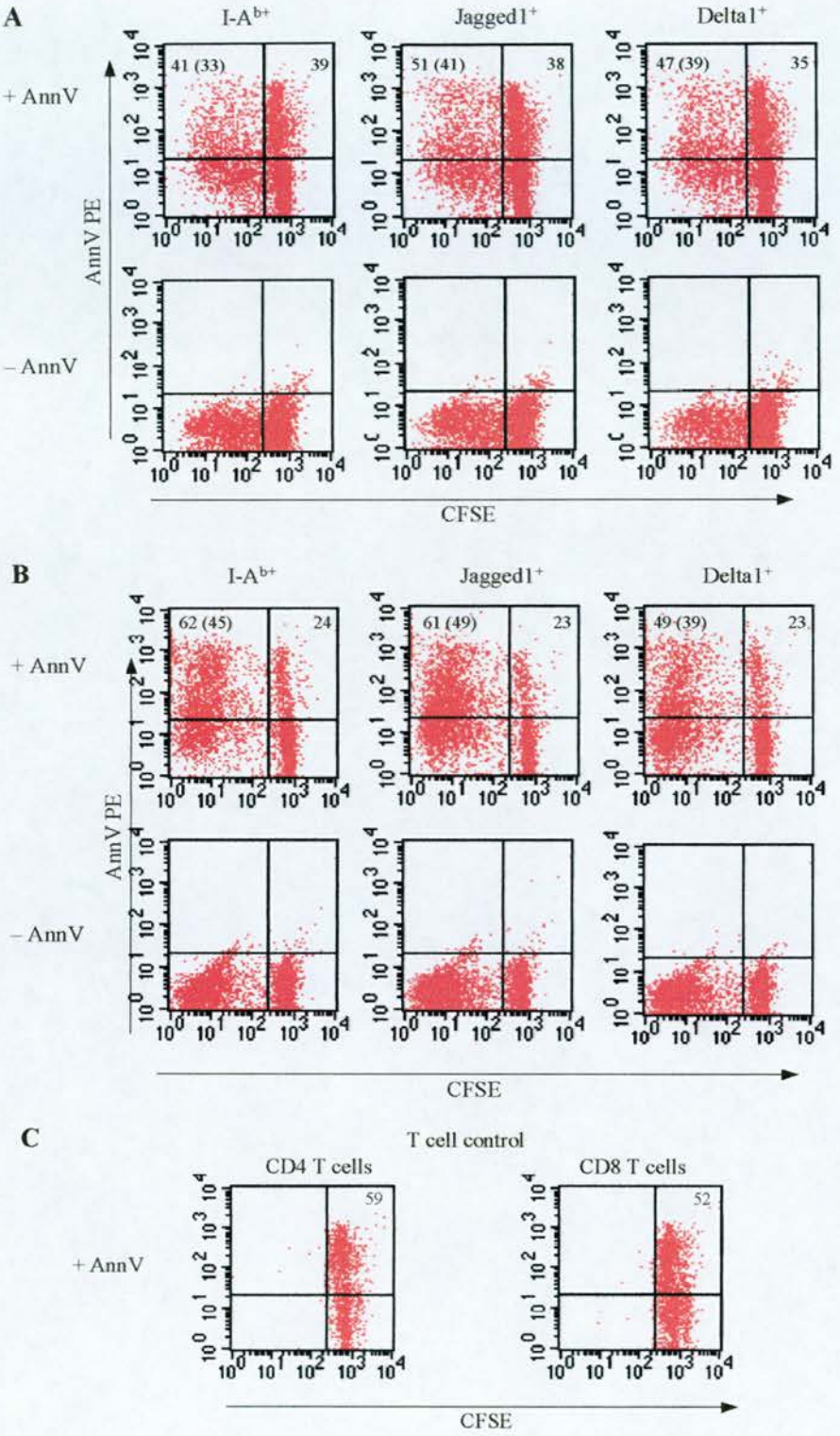

A
I-A^{b+}
Jagged1^{+}
Delta1^{+}
+ AnnV
– AnnV
41 (33)
39
51 (41)
38
47 (39)
35
AnnV PE
CFSE
B
I-A^{b+}
Jagged1^{+}
Delta1^{+}
+ AnnV
– AnnV
62 (45)
24
61 (49)
23
49 (39)
23
AnnV PE
CFSE
C
T cell control
CD4 T cells
CD8 T cells
+ AnnV
59
52
CFSE

Figure V.14. Downregulation of IFNγ produced by T cells in MLR with Jagged1$^+$ L cells is not due to increased cell death of proliferating T cells.
A MLR was carried out with mitomycin C-treated Jagged1$^+$, Delta1$^+$ or I-A^{b+} L cells as stimulators and CFSE-labelled T cells which were enriched from BALB/c spleens by nylon wool purification. Control T cells were incubated in medium. After 5 days, cells were stained for CD4 or CD8 and Annexin V and analysed by flow cytometry as described in section II.3.9. CD4$^+$ or CD8$^+$ T cells were gated as seen previously.
[A] & [B], Dot plot analysis with or without PE-conjugated Annexin V (FL2, +/– AnnV) and CFSE (FL1) of gated CD4$^+$ or CD8$^+$ T cells ([A] and [B], respectively) of the MLRs (Jagged1$^+$, Delta1$^+$ and I-A^{b+}).
[C] Dot plot analysis with Annexin V (+ AnnV) and CFSE of gated control CD4$^+$ and CD8$^+$ T cells. Quadrant grid was set using control T cells for FL1 and MLR without Annexin V staining for FL2. Numbers within the left and the right upper quadrant represent percentage of Annexin V$^+$ cells of the proliferating population (CFSE^{low}) and the resting population (CFSE^{high}), respectively. Numbers in parenthesis represent percentage of Annexin V$^+$ cells of the entire population.
Details of antibodies are given in table II.4 & II.5.

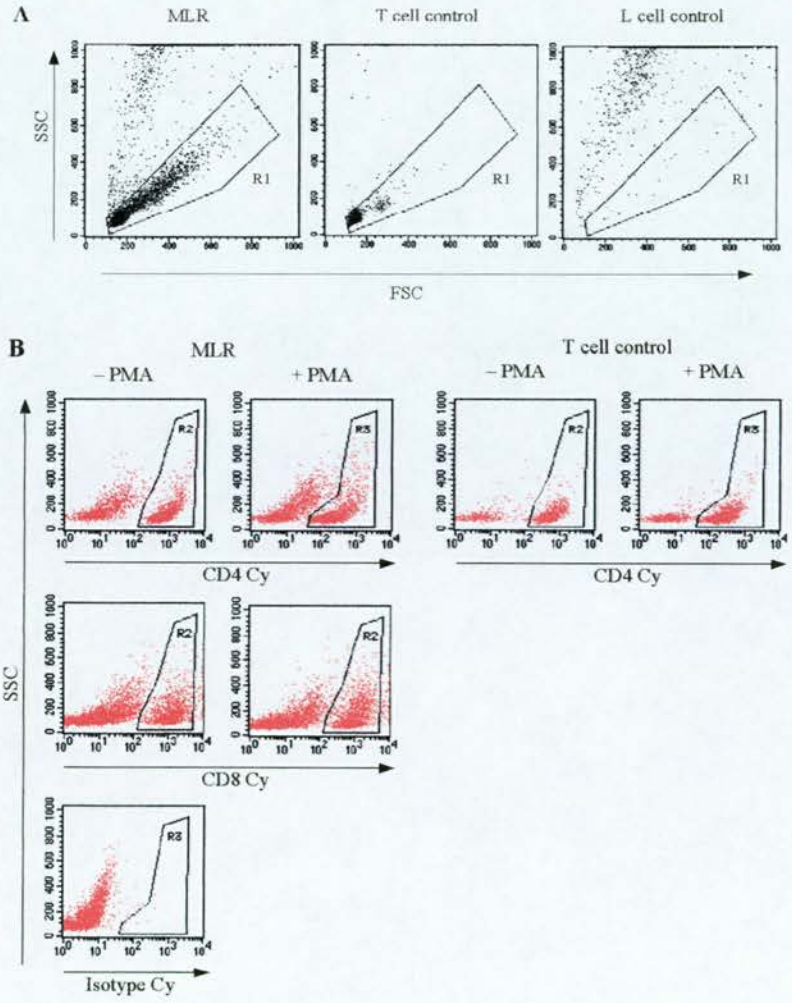
V.9 Jagged1 does not decrease numbers of IFNγ secreting T cells activated in a MLR

Similar numbers of IFNγ producing T cells are present in MLRs with Jagged1$^+$, Delta1$^+$ and I-A^{b+} L cells.

Supernatant of MLRs with Jagged1$^+$ L cells contained less IFNγ than those of MLRs with Delta1$^+$ or I-A^{b+} L cells. It is of interest to see whether this was also reflected at the level of intracellular IFNγ. MLRs were carried out with nylon wool-enriched T cells. After 4 days, the cells were activated with PMA and ionomycin for 4 hours with monensin-containing GolgiStop added to the final three hours. Adding PMA/ionomycin to the cultures caused a decrease of the cell surface expression of CD4 but not CD8 in activated (MLR) and non-activated cells (T cell control) (Fig. V.15B). A decrease in CD4 expression following incubation with PMA alone or in conjunction with ionomycin has been reported previously (Pelchen-Matthews *et al.*, 1993). However, there was still a good separation between CD4$^+$ and CD4$^-$ cells because the CD4 antibody used was conjugated to Cy-Chrome, which is a bright fluorochrome.

Numbers of IFNγ-containing cells were low and did not change in PMA/ionomycin-activated $CD4^+$ T cells present in the different MLRs (Fig. V.15C). Interestingly, resting $CD4^+$ T cells (control T cells) had the same number of IFNγ-producing cells after PMA/ionomycin activation. $CD8^+$ T cells had higher amount of IFNγ-containing cells, but again there were no changes between the different cultures (Fig. V.15D). A small percentage of the resting $CD8^+$ T cells (control T cells) also produced intracellular IFNγ after PMA/ionomycin activation.

In conclusion, numbers of IFNγ producing $CD4^+$ or $CD8^+$ T cells did not alter in the presence of the Notch ligands. $CD4^+$ T lymphocytes activated by the L cell transfectants did not contain a higher percentage of IFNγ-producing cells compared to the control $CD4^+$ T cells. This may be explained by the presence of effector $CD4^+$ T cells previously activated *in vivo*, which could rapidly produce IFNγ upon PMA/ionomycin activation.



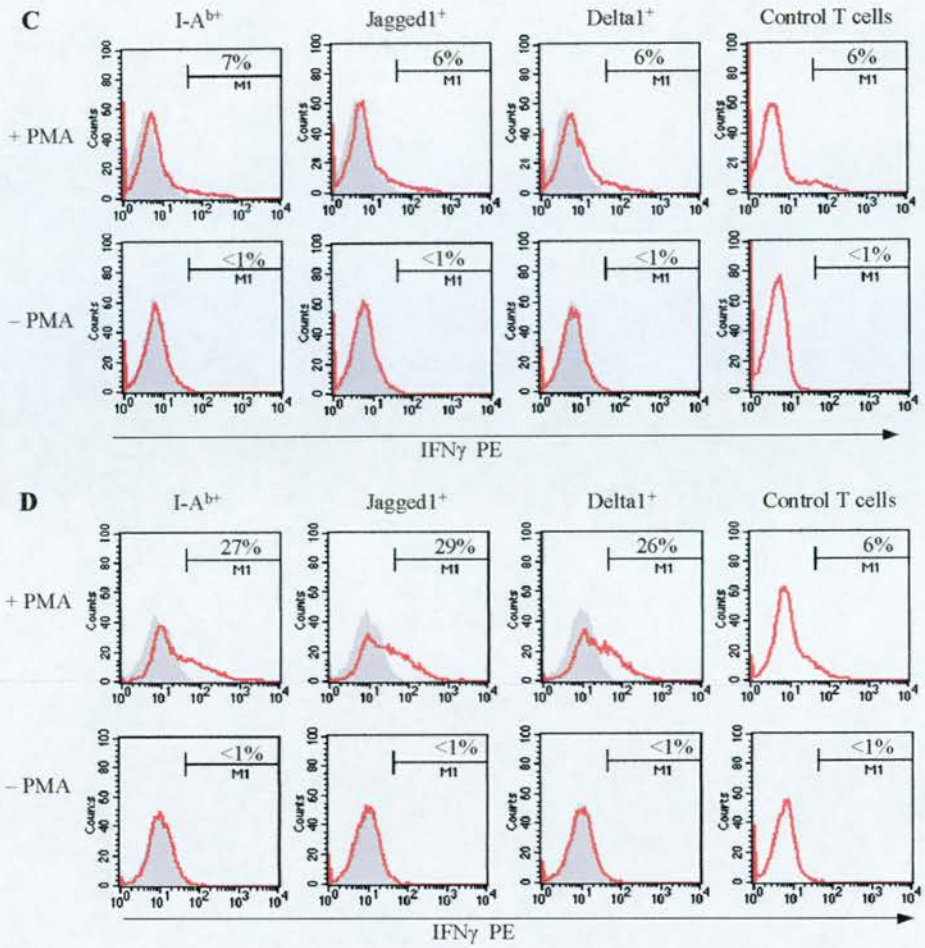


Figure V.15. Similar numbers of IFNγ producing T cells are present in MLRs with Jagged1$^+$, Delta1$^+$ and I-A^{b+} L cells.

A MLR was carried out with mitomycin C-treated Jagged1$^+$, Delta1$^+$ or I-A^{b+} L cells as stimulators and nylon wool-enriched T cells from BALB/c spleens. Control T cells and control L cells were incubated in medium. After 4 days, cells were stimulated with PMA and ionomycin (+PMA) or further kept in media (–PMA) for 4 hrs with GolgiStop added for the final 3 hrs as described in materials & methods, section II.3.8.2. Lympholyte-treated cells were stained for CD4 or CD8 before fixation, permeabilisation and staining for intracellular cytokines.

[A] Forward scatter (FSC) and side scatter (SSC) analysis with lymphocyte gate R1 of a representative MLR, pooled Jagged1$^+$, Delta1$^+$ and I-A^{b+} L cells incubated in medium (L cell control) and T cells incubated in medium (T cell control) are shown.

[B] FL3 (Cy-Chrome) and side scatter (SSC) analysis of R1-gated cells stained with anti-CD4, anti-CD8 or isotype control. Region R2 was used to gate for CD8$^+$ T cells and for non-stimulated CD4$^+$ T cells and region R3 for PMA/ionomycin-stimulated CD4$^+$ T cells.

[C] Histograms showing PMA/ionomycin-stimulated cells (+PMA) gated for lymphocyte scatter (R1) and CD4 expression (R3) and non-stimulated cells (–PMA) gated for lymphocyte scatter (R1) and CD4 expression (R2).

[D] Histograms showing PMA/ionomycin-stimulated and non-stimulated cells (+PMA and –PMA, respectively) gated for lymphocyte scatter (R1) and CD8 expression (R2).

Shaded histogram: Isotype control, thick red line: IFNγ. Numbers within the histograms represent percentages within the marker bounds. Markers were set using isotype control stainings.

Details of antibodies are given in table II.4 & II.5.

Numbers of proliferating T cells producing IFNγ is not downregulated in MLR with Jagged1$^+$ L cells.

To distinguish *in vivo* activated or memory T cells from T lymphocytes stimulated in the MLRs, cells were CFSE-labelled prior to activating them with L cells. After 5 days, cells were PMA/ionomycin-activated and analysed as described above (Fig. V.15). Percentages of IFNγ-positive cells of the entire CD4$^+$ population were similar as seen in the previous experiment (Fig. V.16A). Numbers of the alloreactive CD4$^+$ T cells (CFSE^{low}) containing intracellular IFNγ did not differ between the MLRs with the different L cell lines. Percentages of IFNγ-producing CD8$^+$ T cells were slightly higher in response to Jagged1$^+$ and I-A^{b+} L cells than in response to Delta1$^+$ L cells after PMA/ionomycin activation (Fig. V.16B). Interestingly, some alloreactive CD8$^+$ T cells produced IFNγ even in the absence of additional activation (–PMA). Double the number of IFNγ$^+$ CD8$^+$ T cells were found in the culture with Jagged1$^+$ L cells compared to the culture with I-A^{b+} L cells. This observation was consistently seen. Intracellular IL-4 was not detected in proliferating CD4$^+$ T cells (data not shown).

These data show that the number of T cells producing intracellular IFNγ was not decreased in response to Jagged1 expressing L cells. If anything, percentage of IFNγ$^+$ CD8$^+$ T cells activated by Jagged1$^+$ L cells seemed to be higher in the absence of PMA/ionomycin.

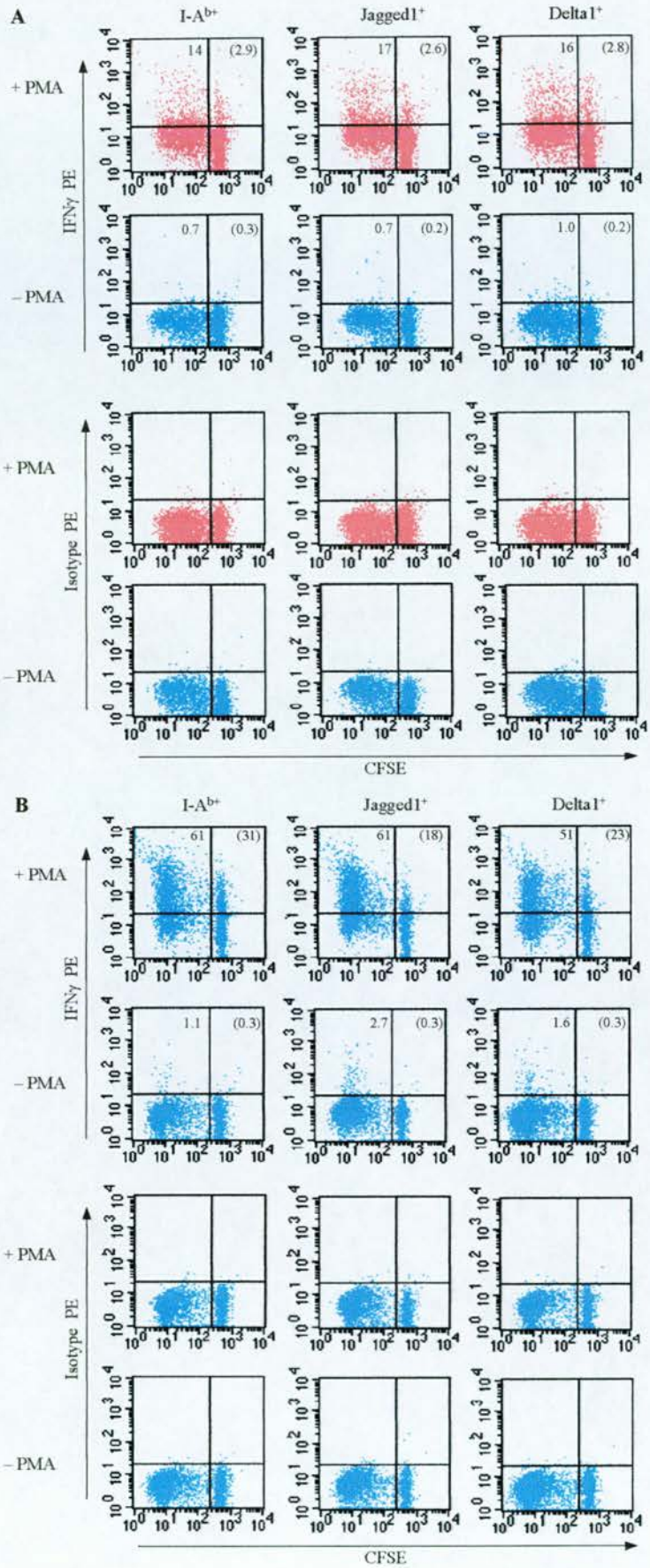

A
I-Ab+
Jagged1+
Delta1+
+ PMA
– PMA
IFNγ PE
Isotype PE
14 (2.9)
17 (2.6)
16 (2.8)
0.7 (0.3)
0.7 (0.2)
1.0 (0.2)
CFSE
B
61 (31)
61 (18)
51 (23)
1.1 (0.3)
2.7 (0.3)
1.6 (0.3)

Figure V.16. Numbers of proliferating T cells producing IFNγ is not downregulated in MLR with Jagged1$^+$ L cells.
A MLR was carried out with mitomycin C-treated Jagged1$^+$, Delta1$^+$ or I-A^{b+} L cells as stimulators and nylon wool-enriched T cells from BALB/c spleens. Control T cells were incubated in medium. After 4 days, cells were stimulated with PMA and ionomycin (+PMA) or further kept in media (–PMA) for 4 hrs with GolgiStop added for the final 3 hrs as described in materials & methods, section II.3.8.2. Lympholyte-treated cells were stained for CD4 or CD8 before fixation, permeabilisation and staining for intracellular cytokines. The gates for lymphocyte scatter and CD4/CD8 expression were set as previously shown (Fig. V.15).
[A] Dot plots showing PMA/ionomycin-stimulated cells (+PMA) gated for lymphocyte scatter (R1) and CD4 expression (R3, pink dots) and non-stimulated cells (–PMA) gated for lymphocyte scatter (R1) and CD4 expression (R2, blue dots).
[B] Dot plots showing PMA/ionomycin-stimulated and non-stimulated cells (+PMA and –PMA, respectively) gated for lymphocyte scatter (R1) and CD8 expression (R2, blue dots). Quadrant grid was set using control T cells for FL1 and PE isotype control staining for FL2.
Numbers within the dot plots represent percentage of IFNγ$^+$ cells of the proliferating population (CFSE^{low}) and (percentage of IFNγ$^+$ cells of the resting population (CFSE^{high})).
Details of antibodies are given in table II.4 & II.5.

V.10 Discussion

I and other investigators have shown that Notch receptors, ligands and downstream components are present in T lymphocytes and APCs (chapter III & IV; Hoyne *et al.*, 2000; Ng *et al.*, 2001) and their expression is dependent on the activation or maturation state of the T cells and APCs, respectively. Further, overexpression of Jagged1 on APCs downregulates an immune response *in vivo* (Hoyne *et al.*, 2000). It is, therefore, of interest to investigate whether the Notch ligands expressed on APCs influence the induction of an immune response *in vitro*. Murine fibroblasts co-transfected with I-A^b and either human Jagged1 or murine Delta1 were used as APCs in MLRs. This study demonstrates that Jagged1 and Delta1 can differentially signal Notch expressed on T lymphocytes and that Jagged1 expressed by the L cell transfectants downregulates secretion but not production of IFNγ by the T cells. These results support the idea that Notch signalling plays an important role in modelling an immune response.

Detection of components of the Notch pathway in the cells of the immune system at the protein level is difficult due to lack of specific antibodies. In a transfection system, however, detection of transcripts of the transfected molecules is not sufficient to assure protein expression, as there is no guarantee that translation will occur. Neither does it allow selection of the transfected cells. Therefore, the pIRES2-EGFP vector (Clontech Laboratories) was used to co-transfect the I-A^{b+} L cells with human Jagged1 or murine Delta1. The gene of interest (Jagged1 or Delta1) was placed upstream of the internal ribosome entry site (IRES) and the reporter gene EGFP (Crameri *et al.*, 1996). Both genes were transcribed as a single bicistronic mRNA (Rees *et al.*, 1996). EGFP, situated downstream of Jagged1 or Delta1, was translated by re-entry of ribosomes at the IRES. Thus, EGFP-positive cells were expected to express Jagged1 or Delta1 as well. This allowed identification of Jagged1 or Delta1 expressing cells as an EGFP-positive cell population by flow cytometry. A highly purified population of L cells expressing EGFP was obtained by FACS sorting (Fig. V.1A).

Expression of the gene of interest (human Jagged1 or murine Delta1) was confirmed by RT-PCR and real-time PCR analysis (Fig. V.2 & V.3). Even though I-A^{b+} L cells expressed low levels of endogenous Jagged1 and Delta1, mRNA levels of the transfected molecules increased greatly. In the case of Delta1^{+} L cells the increase was over 3000-fold. Jagged2 and Delta3 were slightly upregulated in Jagged1 expressing L cells. This did not seem to be an artefact of real-time PCR because the upregulation was reproducible even after culturing the L cells for several weeks in their appropriate selection medium. Expression of Notch receptors 1 and 3 did not differ between the different L cell lines. Several studies have revealed that Notch ligands are able to activate the Notch pathway in an autocrine fashion (Doherty *et al.*, 1996; Wong *et al.*, 2000). Since Notch receptors were present in L cells, expression of downstream elements of the Notch pathway were also analysed. Expression of Hes1 did not alter between the different L cell lines. There was no detectable expression of Hes5 in L cells. Deltex1 was expressed only upon co-transfection of I-A^{b+} L cells with Jagged1. It is known that Notch1 activation can lead to upregulation of Deltex1 transcript (Deftos *et al.*, 1998; Deftos *et al.*, 2000). Thus overexpression of Jagged1 in the L cells may activate Notch signalling and lead to induction of Deltex1 transcription.

There is conflicting data concerning which molecules are capable of providing costimulation for primary proliferative T cell responses. In part the confusion is the result of drawing conclusions from different *in vitro* systems and from the use of different responder T cell populations. Cross-linking the TCR in the presence of anti-CD28 antibodies induces a high

proliferative responsive and cytokine production (Fig. III.2 & III.4; Baroja *et al.*, 1989). In MLRs, B7.1 has been shown to provide sufficient costimulation to activate alloreactive T cells (Galvin *et al.*, 1992). These responses are inhibited by blocking B7 interactions (Freeman *et al.*, 1993). In some cases, accessory molecules appear to be sufficient to induce T cell responses in MLRs (Wakkach *et al.*, 2001). However, their requirement is not absolute but may augment the T cell response (Damle *et al.*, 1992; Hargreaves *et al.*, 1995).

All three lines of transfected L cells expressed high levels of the MHC class II molecule I-A^b and moderate levels of the MHC class I molecule H-$2K^k$ and the costimulatory molecule B7.1. Even though ICAM-1 was not expressed (data not shown), the L cells were capable to activate T cells in a MLR confirming data by Altmann and colleagues that high levels of MHC molecules can compensate for the lack of ICAM-1 (Altmann *et al.*, 1989). In summary, phenotyping of the L cells at the RNA and protein levels did not reveal any differences between the three different L cell lines with exception of induced Deltex1 expression in $Jagged1^+$ L cells.

As expected, I-A^{b+} L cells induced an alloresponse in both $CD4^+$ and $CD8^+$ T cells isolated from mice expressing the H-2^d haplotype, whereas untransfected L cells activated $CD8^+$ but not $CD4^+$ T cells of the H-2^d haplotype (Fig. V.5). The increased proliferation of $CD8^+$ T cells observed with the I-A^{b+} L cells compared to untransfected L cells may be due to the higher background proliferation of the L cells. Another explanation is the existence of additional peptide determinants derived from the transfected I-A^b molecule: Many self-peptides presented by class II molecules originate from class II molecules (Fedoseyeva *et al.*, 1996; Murphy *et al.*, 1992; Rudensky and Janeway, 1993) and are recognised by alloreactive T cells (de Koster *et al.*, 1989; de Koster *et al.*, 1992; Weber *et al.*, 1995). Further it has been reported that certain peptides can be presented by both class I and class II molecules (Hickling *et al.*, 1990). It is also known that soluble proteins can be transported into the cytosol, processed by the proteosome and subsequently presented by class I molecules (Albert *et al.*, 1998b; Androlewicz, 2001). Additionally, it has been reported that CTL can recognise determinants from I-A^b molecules (de Waal *et al.*, 1981). It is, therefore, possible that peptides of the transfected I-A^b molecule can be presented in context with class I and thereby increasing allogenicity of the I-A^{b+} L cells to $CD8^+$ T cells compared to the untransfected L cells.

Variation in levels of proliferation within and between experiments was observed most probably due to the incomplete inhibition of L cell proliferation and this influenced cytokine secretion. However, complete inhibition of L cell growth obtained with different methods, such

as fixation or overnight incubation of the L cells in mitomycin C, rendered the L cells incapable to activate T cells in a MLR (data not shown). Even though the amount of proliferation and IFNγ production varied, the trend of less IFNγ secreted by T cells in response to Jagged1$^+$ L cells was consistent and confirmed by statistical analysis.

Interestingly, purified CD4$^+$ T cells in response to I-A^{b+} or Delta1$^+$ L cells produced IFNγ at levels of up to 600pg/ml (Fig. V.6B). Again from comparing the results of different experiments, marked variation in levels of IFNγ secreted was observed. However, this did not correlate with the magnitude of proliferation. The response of CD8$^+$ T cells in MLRs was similar (Fig. V.7, Experiment #1 vs. Experiment #2). There are several explanations that may account for these findings. In general, naïve CD4$^+$ T cells are believed to produce high levels of IFNγ after their differentiation into T_H1 cells (Murphy *et al.*, 2000). Differentiation into either T_H1 or T_H2 usually requires restimulation of the CD4$^+$ T cells. However, in this study, CD4$^+$ T cells were activated in a primary MLR. It is, therefore, possible that positive selection of CD4$^+$ T cells by MACS purification induced an additional signal, which added to the stimulation by the L cells. In a few cases, MACS purification was sufficient to induce moderate proliferation of CD4$^+$ T cells and high IFNγ production (data not shown). The protein tyrosine kinase Lck of the Src family is non-covalently associated with the CD4 molecule (Fig. I.4, section I.3). Upon TCR activation Lck rapidly phosphorylates ITAM motifs of the CD3 cluster, which permits TCR association with the Syk family kinase ZAP-70 (Chan *et al.*, 1992; Straus and Weiss, 1992). These events are crucial for the triggering of the signalling cascades that stimulate nuclear transcription factors that regulate the production of several cytokines. Cross-linking of CD4 by MACS beads may, therefore, facilitate cytokine induction. Additionally, the CD4 molecule has been proposed to act as an IL-16 receptor because it is an absolute requirement for signalling and functional responses to IL-16 (Cruikshank *et al.*, 1987; Cruikshank *et al.*, 1994). IL-16 increases expression of IL-2Rα (CD25) and IL-2Rβ on CD4$^+$ T cells and in concert with IL-2 increases proliferation and production of IFNγ (Parada *et al.*, 1998). Cross-linking of the CD4 molecule may, therefore, induce activation events similar to IL-16 signalling.

Even though IFNγ was detected in the supernatant of CD4$^+$ T cells cocultured with L cells, transcripts for IFNγ were only marginally increased (Fig. V.10). However, a different method of T cell purification was used for this experiment: instead of positively selecting CD4$^+$ T cells by MACS, the T cells were enriched by nylon wool purification. After their activation in the MLR, CD4$^+$ and CD8$^+$ T cells were collected by FACS sorting. Thus, the CD4 molecules

were never cross-linked by MACS beads, which may explain the low induction of IFNγ. Additionally, FACS sorting yielded cell populations of high purity (>99%) in contrast to MACS isolation (95±2% for $CD4^+$ T cells). A few contaminating $CD8^+$ T cells in the MACS-purified $CD4^+$ T cell prep could increase the IFNγ content in the supernatant of the MLR. $CD4^+$ T cells, known to produce a higher level of IL-2 than $CD8^+$ T cells, would provide IL-2 as a costimulation signal for the $CD8^+$ T cells.

Naïve $CD8^+$ T cells do not necessarily require costimulation for proliferation or effector function (Wang *et al.*, 2000). Activation through TCR engagement without any costimulation induced effector function (proliferation and cytotoxicity) in $CD8^+$ T cells within 48 hours. It also has been reported that $CD8^+$ T cells are able to lyse L cells in a MLR (Reiss *et al.*, 1983). Thus, CTL-mediated killing of L cells could also influence activation of $CD4^+$ T cells at a later stage.

IFNγ production in a MLR is dependent on IL-2 release because secretion of IFNγ was almost completely inhibited after anti-IL-2 treatment (Danzer *et al.*, 1994). IL-2 was detected after 72 hours in the supernatant of MLRs with L cells and nylon wool enriched T cells (Fig. V.8C). Levels of IL-2 in the supernatant correlated in general with the amount of proliferation.

IL-2 transcription was induced at similar level in $CD4^+$ T cells activated in the MLR with $Jagged1^+$, $Delta1^+$ and $I\text{-}A^{b+}$ L cells as shown by real-time PCR (Fig. V.10). Since it has been reported that already small amounts of IL-2 seemed to be sufficient to for maximal IFNγ release in a MLR (Danzer *et al.*, 1994), I concluded that lack of IL-2 production was not responsible for the decreased IFNγ secretion in response to $Jagged1^+$ L cells.

In chapter III it has been shown that addition of IL-10 to anti-CD3/CD28-activated $CD4^+$ T cells reduced the levels of transcripts and protein for both IL-2 and IFNγ on protein and RNA level. It is, therefore, possible that IL-10 produced by T cells in response to $Jagged1^+$ L cells is acting autocrinally causing a decrease in IFNγ production. Some investigators have failed to detect IL-10 in MLRs (Danzer *et al.*, 1994) whereas others have reported its presence (Toungouz *et al.*, 1996). Toungouz and colleagues found that the mismatch of one MHC class II molecule induced the maximal amount of IL-10 secretion. The transfected $I\text{-}A^b$ molecule on MHC class II-negative L cells represents such a single mismatch to $CD4^+$ T cells from a $H\text{-}2^d$ background. However, IL-10 was not detected by ELISA at any time point of the MLR. This

may be due to detection limits of the assay. Blocking antibodies to the IL-10 receptor did not increase the production of IFNγ in response to any of the L cells (data not shown). The absence of IL-10 may also reflect that L cells expressing class II are not the same as conventional APCs; for instance they do not express ICAM-1. Real-time analysis of $CD4^+$ T cells activated by the L cells revealed a small increase of IL-10 compared to non-activated $CD4^+$ T cells, but no differences were found in response to stimulation with the different L cell transfectants (Fig. V.10). Therefore, it was concluded that IL-10 was not responsible for the downregulation of IFNγ secretion by T cells in response to $Jagged1^+$ L cells.

While interactions between CD28 and members of the B7 family costimulate and enhance T cell responses, recent evidence indicates that the CD28 homologue CTLA-4 plays a role in downregulating responses. CTLA-4 can compete for CD28/B7 interactions and thus deliver negative signals resulting in T cell anergy (Thompson and Allison, 1997). Naïve T cells were rendered hyporesponsive to specific allogeneic antigen after coculturing with MHC-mismatched stimulators in the presence of CTLA-4-Ig (Lee *et al.*, 2001), a soluble fusion protein of human CTLA-4 and the Fc region of IgG1 capable of blocking T cell activation *in vitro* (Lenschow *et al.*, 1992). These antigen-specific hyporesponsive T cells were subsequently able to actively inhibit the allogeneic responses of naïve syngeneic T cells in a cell-to-cell contact-dependent manner (Lee *et al.*, 2001). B7.1 has been identified as the dominant ligand for CTLA-4-mediated downregulation of alloimmune responses *in vivo* (Yamada *et al.*, 2001). CTLA-4 is stored mainly in intracellular compartments (Linsley *et al.*, 1996). Induction of the secretion of CTLA-4 may consequently downregulate the immune response. Therefore, CTLA-4 upregulation after coculturing the T cells with Jagged1 or Delta1 expressing L cells was investigated. However, CTLA-4 was not detected on the cell surface of activated $CD25^+CD4^+$ T cells (Fig. V.11C) nor on proliferating $CFSE^{low}$ $CD4^+$ T cells (Fig. V.12C). This is in contrast to the results of Walunas *et al.* who reported that expression of CTLA-4 was upregulated 2 to 3 days following T cell activation (Walunas *et al.*, 1994). Furthermore regulatory $CD4^+CD25^+$ T cells were found to express CTLA-4 constitutively (Read *et al.*, 2000; Takahashi *et al.*, 2000). However, Read *et al.* were using permeabilised $CD4^+$ T cells and therefore, detected intracellularly stored CTLA-4. Additionally, it was proposed that CTLA-4 affect a secondary rather than a primary immune response (Chambers *et al.*, 1996). In conclusion, CTLA-4 activation was not considered to be responsible for the downregulation of IFNγ in T cells activated by $Jagged1^+$ L cells.

Apoptosis of activated T cells is an important method of downregulating potentially destructive T cells responses (Van Parijs and Abbas, 1996). Mechanisms by which this occurs include Fas-induced cell death and apoptosis induced by a lack of appropriate cytokines (Russell, 1995). TCR-dependent apoptosis in T cell lines requires the expression of members of the Nur77 orphan nuclear hormone receptor family (Liu *et al.*, 1994; Woronicz *et al.*, 1994). It has been shown that Notch1 interacts physically with Nur77 and that Notch1 protects T cell lines from Nur77-dependent T cell death (Jehn *et al.*, 1999). On the other hand, a constitutively active form of Notch1 induced cell cycle arrest accompanied by apoptosis in a B cell line (Morimura *et al.*, 2000). Here, I have shown that Jagged1 and Delta1 expressed on L cells could activate Notch signalling in T cells during the MLR as both ligands induced transcription of Hes1 (Fig. V.10). It was, therefore, of interest to examine if Notch signalling has any influence on T cell survival since this may explain the observed downregulation of IFNγ in the MLR with Jagged1$^+$ L cells. O'Flaherty and colleagues reported that T cells were resistant to apoptosis for the first 5 days after activation in a MLR even though they rapidly acquired expression of Fas (O'Flaherty *et al.*, 2000). The maximal apoptotic response was observed after 7 days. In this study, apoptosis was measured at day 5 in order to forestall the maximal rate of cell death in a MLR. Jagged1 expressing L cells did not induce increased apoptosis in CD4$^+$ or CD8$^+$ T cells compared to I-A^{b+} L cells (Fig. V.13 & V.14). Interestingly, Annexin V binding increased progressively as CD4$^+$ T cells were dividing (Fig. V.14A), which is consistent with data showing that apoptotic cells were generated after each cell division in cultured cell lines (Usherwood *et al.*, 1999). Since no reproducible differences in T cell death were observed between the MLRs stimulated with the different transfected L cell lines, it was concluded that neither Delta1 nor Jagged1 signalling influenced T cell survival.

Both Jagged1 and Delta1 induced upregulation of Hes1 in CD4$^+$ T cells, but only Jagged1 downregulated secretion of IFNγ. The different outcome of Notch signalling can be explained in several ways. Delta1 induced Hes1 transcription in CD4$^+$ but not CD8$^+$ T cells. Furthermore, the Delta1-mediated upregulation of Hes1 was over twofold less compared to Jagged1. It has been shown that relatively minor changes in the dosage of Notch signalling induced phenotypic effects during invertebrate development (Artavanis-Tsakonas *et al.*,

1995). Different levels of Hes1 induction may, therefore, be responsible for the distinct result of Jagged1- and Delta1-mediated Notch signalling. Alternatively, modulators of Notch signalling such as Fringe may alter the outcome of Notch activation (Hicks *et al.*, 2000; Panin *et al.*, 1997).

There was an apparent discrepancy between downregulation of secreted IFNγ and levels of transcripts, which were only minimally decreased in T cells responding to Jagged1$^+$ L cells as compared to I-A^{b+} L cells. Therefore, I concluded that Notch signalling is not solely acting on IFNγ transcription. Regulation of protein expression can occur at several stages: The most obvious is transcription, which was not the case in this study. Post-transcriptional regulation is quite common in cytokine expression (Lindsten *et al.*, 1989; Waldmann and Tagaya, 1999). CD28 signalling stabilises IL-2 mRNA post-transcriptionally (Umlauf *et al.*, 1995). If the reason for the downregulation of secreted IFNγ lies in post-transcriptional regulation, it should also been seen in the amount of intracellular residing IFNγ.

Based on an adaptation of the fixation and permeabilisation method described by Pala and colleagues (Pala *et al.*, 2000), intracellular residing IFNγ and IL-4 was measured by flow cytometry. An advantage of this method is that it allows analysis of IFNγ-producing cells on a single cell basis in contrast to real-time PCR or ELISA, which measure IFNγ production of an entire cell population. Interestingly, the downregulation of IFNγ measured by ELISA was not detected by intracellular cytokine staining of the entire CD4$^+$ or CD8$^+$ T cell population (Fig. V.15). Control CD4$^+$ T cells incubated in medium showed a similar frequency of IFNγ-containing cells than CD4$^+$ T cells activated in the MLR. This is most probably due to effector CD4$^+$ T cells, which were previously activated *in vivo*. Upon PMA/ionomycin stimulation they were capable of producing IFNγ. Intracellular IL-4 was not detected consistent with results obtained by ELISA.

To exclude non-specific effector T cells, I made use of CFSE, a fluorescent dye, which allows the tracking of dividing cells by flow cytometry (described in materials & methods, II.3.7). Resting cells did not loose fluorescence. Therefore, they can be identified as the tight cell population with the highest fluorescence in the FL1 channel (Fig. V.14C). In the MLR culture, the resting cells corresponded to the peak with highest level of fluorescence (CFSE^{high}). Dividing cells show an almost exact two-fold decrease in fluorescence from peak to peak (Lyons and Parish, 1994). In the MLR with L cells, proliferating T cells showed a loss of fluorescence but not in clear two-fold steps. This is due to the heterogeneous

population of alloreactive T cells displaying a TCR repertoire with various specificities and affinities (Obst *et al.*, 1998). It is also possible that some of the alloreactive T cells were antigen-experienced: It has been suggested that a particular TCR is not only specific for certain allogeneic MHC molecules but additionally can crossreact with a foreign antigenic peptide plus self-MHC molecule (Obst *et al.*, 1998; Sredni and Schwartz, 1981). Antigen-experienced T cells are known to display effector function such as proliferation much more rapidly than naïve T cells (Garcia *et al.*, 1999; Veiga-Fernandes *et al.*, 2000). Activation of a mixture of antigen-experienced and naïve T cells labelled with CFSE would result in a smeared pattern rather than a multi-peak profile of proliferating T cells. It also has to be noted that many groups using CFSE to track cell division used either TCR-transgenic T cells (de St Groth *et al.*, 1999; Dubois *et al.*, 1998) or polyclonal stimuli such as anti-CD3/CD28 or LPS (Gett and Hodgkin, 1998; Lyons and Parish, 1994).

By gating on proliferating ($CFSE^{low}$) T cells, the frequency IFNγ-producing cells within the alloantigen-specific population can be measured. Again, there was no decrease in the frequency of IFNγ-producing alloreactive $CD4^+$ or $CD8^+$ T cells in response to $Jagged1^+$ L cells (Fig. V.16). Levels of intracellular IFNγ expressed as the mean fluorescence did not differ neither between the alloreactive $CD4^+$ or $CD8^+$ T cells stimulated in the different MLRs (data not shown). The standard protocol for measuring intracellular cytokines requires a four-hour stimulation with PMA/ionomycin. PMA directly activates protein kinase C (PKC) and together with the calcium ionophore ionomycin induces transcription factors NF-AT, AP1 and NF-κB, which are required for IL-2 gene transcription (section I.3; Kuo and Leiden, 1999).

It cannot be excluded that activation with PMA/ionomycin might have negated signals of the Notch pathways. However, other groups found Notch-mediated inhibition of NF-AT/AP1 promoter activity in Jurkat T cells stimulated with anti-TCR or PMA/ionomycin (Izon *et al.*, 2001). Additionally, there is accumulating evidence that the activated form of Notch interacts physically with NF-κB, which is downstream of PKC. Activated Notch1 inhibits NF-κB-dependent transcription in a similar fashion as IκB (Guan *et al.*, 1996; Wang *et al.*, 2001). Further it has been reported that Notch signalling is capable to inhibit the expression of certain cytokines: The human Notch intracellular domain inhibits IL-8 expression in a human erythroleukemic cell line (Lam *et al.*, 2000) and CBF1 inhibits IL-6 expression by competing for NF-κB site on the IL-6 promoter (Palmieri *et al.*, 1999). Notch may also influence events via Deltex1, which has been shown to inhibit Ras signalling (Ordentlich *et al.*, 1998).

In conclusion, Jagged1 and Delta1 expressing L cells induced Notch signalling leading to increased expression of Hes1 in T cells. T cells activated in a MLR with Jagged1$^+$ L cells produced similar levels of intracellular IFNγ compared to T cells in response to Delta1$^+$ and I-A^{b+} L cells. However, IFNγ was significantly reduced in the supernatant of MLRs with Jagged1$^+$ L cells. The reduction of secreted IFNγ was not due to inhibition of transcription nor translation of IFNγ because the level of intracellular IFNγ was not reduced.

There are several ways to explain this observation: Jagged1-induced Notch signalling may inhibit secretion of IFNγ. This inhibition would need to be specific for the secretion of IFNγ, since it was not observed for IL-2. Cytokine secretion and division number is independent of time for IL-2, IL-3, IL-4 and IL-10: For example, CD4$^+$ T cells having divided six times produced the same amount of IL-4 on day 4 as on day 5 measured by either ELISA or intracellular cytokine staining (Gett and Hodgkin, 1998). However, time appeared to have an effect on levels of IFNγ in supernatants but not when measured by the intracellular staining method: CD4$^+$ T cells having divided six times secreted a higher level of IFNγ on day 4 than on day 5. Nonetheless, the level of intracellular IFNγ of CD4$^+$ T cells after 6 divisions did not differ between day 4 or day 5. The authors suggested an additional level of control for IFNγ secretion involving unknown factors inhibitory to IFNγ release. Thanhäuser *et al.* also suggested a post-transcriptional regulation of IFNγ release: They found that the phosphodiesterase inhibitor pentoxifylline blocked secretion but not transcription of IFNγ and IL-2 in phytohaemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs) (Thanhauser *et al.*, 1993).

The secretory lysosomes found in haemopoietic cells provide a very efficient mechanism for delivering the effector proteins of many immune cells in response to antigen recognition. Although haematopoietic cells differ from conventional secretory cells (e.g. neuroendocrine), they express proteins important for the tight regulation of secretion (Stinchcombe and Griffiths, 2001). The best evidence that unique components in haematopoietic cells are involved in secretory lysosome exocytosis has come from the autosomal recessive disease Chediak-Higashi syndrome (Ward *et al.*, 2000). The beige mouse model of this disease lacks T- and NK-cell activity. The defect is neither an absence of T and NK cells, nor their ability to produce lytic proteins, but rather their inability to secrete their lytic granules (Baetz *et al.*, 1995). Another example of regulated secretion process in T lymphocyte is the upregulation of CTLA-4 from storage compartments upon T cell activation (Linsley *et al.*, 1996).

In conclusion, T lymphocytes are capable of regulating secretion and therefore, Notch may interfere with this regulation by an unknown mechanism. Interestingly, it has been proposed that Notch can regulate the actin cytoskeleton (Lowell and Watt, 2001; Zecchini *et al.*, 1999), which plays an essential role in forming the 'immunological synapsis' and the polarisation of the secretory apparatus of the T cells towards contact site (Acuto and Cantrell, 2000). To investigate whether Notch activation can inhibit secretion of IFNγ, intracellular IFNγ could be measured in the absence of secretion inhibitors such as Brefeldin A or monensin. An increase in intracellular IFNγ should occur in PMA/ionomycin-activated and non-activated T cells cocultured with Jagged1 expressing L cells.

Another possibility to explain the downregulation of IFNγ in the supernatant of MLRs with Jagged1$^+$ L cells would involve an increased uptake of IFNγ by the T cells resulting in reduced extracellular IFNγ. The IFNγ receptor is a heterodimer consisting of an α and a β chain (reviewed in Bach *et al.*, 1997). The α chain is thought to be expressed constitutively, whereas β chain expression can be regulated either positively (e.g. by PMA/ionomycin activation of T cells) or negatively (by IFNγ exposure) in a stimulus-specific manner. Further experiments to address these issues may involve analysis of expression of the IFNγ receptor. Notch-induced upregulation or inhibition of the downregulation of IFNγRβ may account for the decreased level of extracellular IFNγ due to more efficient IFNγ uptake.

A third, less likely explanation is the effect of IFNγ on the L cells. IFNγ induces formation of a specific transcription factor, which appears to co-ordinately upregulate transcription of the genes encoding the class I α-chain, β_2-microglobulin, subunits for the proteasome (LMP) and the transporter (TAP) (Androlewicz, 2001). This facilitates and increases antigen presentation by class I molecules and therefore, represents an amplification loop for activation of T cells by Delta1$^+$ and I-A^{b+} L cells but not by Jagged1$^+$ L cells due to decreased IFNγ in the culture supernatant. Against this explanation stands the fact that at the time of IFNγ release by the T cells, most of the L cells have already died due to the mitomycin C treatment.

VI Transgenic mice with inducible dendritic cell-specific overexpression of murine Delta1 or human Jagged1

VI.1 Introduction

The generation of transgenic mice induced to overexpress either murine Delta1 or human Jagged1 specifically in DCs would provide an useful tool with which to investigate the role of Delta1 and Jagged1 in DC maturation *in vivo* and consequently in their regulation of an immune response.

Targeting of transgenes to specific cells or tissues uses selected promoters to drive the expression of the gene(s) of interest. This approach has provided insights into the biological effector functions and interactions of a large number of proteins. However, transgenesis is not without limitations. Potential problems may occur because the promoters that are used often initiate gene expression *in utero* and drive gene expression in a largely constitutive fashion thereafter. This confounds phenotypic interpretation by superimposing growth and/or development-related abnormalities. This is a major disadvantage if the gene of interest plays an important role during development, as it is the case for the Notch ligands. In an attempt to resolve this problem, transgenic systems have been developed in which gene expression can be externally regulated. Early attempts focused on a variety of approaches including the use of steroid-inducible and metallothionine-based promoter systems (Mader and White, 1993; Palmiter *et al.*, 1982). The disadvantage of these systems in animal models is that they have eukaryotic promoters and induction results in upregulation of endogenous genes with such promoters as well as the transgene. More recent approaches have used *tet*-regulatory systems based on wild-type and mutated tetracycline transactivator fusion proteins (Gossen and Bujard, 1992). Studies have demonstrated that tet-based systems can be externally regulated after direct injection into cardiac tissue (Fishman *et al.*, 1994), transfection into mesangial

cells that are subsequently injected into visceral organs (Kitamura, 1996), and microinjection using standard transgenic technology (Kistner *et al.*, 1996). The "reverse tetracycline (Tc)-controlled transactivator" (rtTA) system where doxycyclin (dox), a tetracycline homologue, acts as an inducer of transcription (Fig. VI.1) can be operated in a quantitative and highly specific way in mice. The kinetics of induction are rapid and it employs a highly regulated and specific prokaryotic promoter system suitable for use in animals (Kistner *et al.*, 1996). The P_{bi-1} promoter contains the Tc-responsive element (TRE), which consists of seven copies of the Tc operator sequence (*tet*O). The TRE element is between two identical minimal cytomegalovirus (CMV) promoters (P_{minCMV}), which lack the enhancer portion that is part of the complete CMV promoter. Consequently, P_{bi-1} is silent in the absence of binding to rtTA to the *tet*O sequences. $P_{minCMV-1}$ controls the expression of the gene of interest, whereas $P_{minCMV-2}$ controls the expression of a reporter gene.

To ensure a DC-specific overexpression of the gene of interest, the rtTA can be put under the control of a DC-specific promoter. For this study, the mouse CD11c promoter was chosen (Brocker *et al.*, 1997) because the expression of CD11c β_2 integrin, as detected by the monoclonal antibody N418 (Metlay *et al.*, 1990), was restricted to mouse DCs in spleen and other tissues such as thymus and lymph nodes (Agger *et al.*, 1990; Brocker, Riedinger and Karjalainen, 1997; Metlay *et al.*, 1990), and therefore represents the best promoter available for murine DC expression. In the bidirectional transcription system (Fig. VI.1) a gene of interest can be indirectly monitored via a reporter gene (Baron *et al.*, 1995). This is especially important if no antibodies exist against the gene of interest, as it is the case for most of the Notch pathway components.

Green fluorescent protein (GFP) is a genetic reporter system derived from the bioluminescent jellyfish *Aequorea victoria* which, when expressed in either eukaryotic or prokaryotic cells and illuminated by blue or UV light, yields a bright green fluorescence (Chalfie *et al.*, 1994; Prasher, 1995). Therefore GFP currently represents a superior alternative to other cell lineage markers in a broad range of applications because it is non-invasive and can hence be viewed in real time *in vitro* or *in situ* in the developing embryo or adult. However, problems encountered with the weak fluorescence and instability of the protein at higher temperatures have generally precluded its use in vertebrate systems. Recently, mutagenesis of the wild-type (wt) gene has yielded several mutant forms, which exhibit greater thermostability and increased fluorescence (Cormack *et al.*, 1996; Heim and Tsien, 1996) overcoming the limitations outlined above (Hadjantonakis *et al.*, 1998; Kato *et al.*, 1999; Okabe *et al.*, 1997). Fluorescence of the enhanced green fluorescent protein (EGFP) is 35-fold stronger than that of wtGFP and the

brightest known GFP variant (Cormack *et al.*, 1996), and it has been codon-optimised for optimal expression in mammalian cells.

I consequently chose the *tet*-regulatory system combined with the DC-specific CD11c promoter and EGFP reporter with which to develop transgenic murine models. Three mouse lines were designed: the CD11c-rtTA line containing the rtTA under the CD11c promoter, the EGFP-Delta1 and the EGFP-Jagged1 lines containing the gene of interest under the P_{bi-1} promoter. By crossing the CD11c-rtTA line with either of the EGFP lines, a transgenic mouse would be generated with the capacity to overexpress EGFP and Delta1 or Jagged1 in DCs following dox feeding.

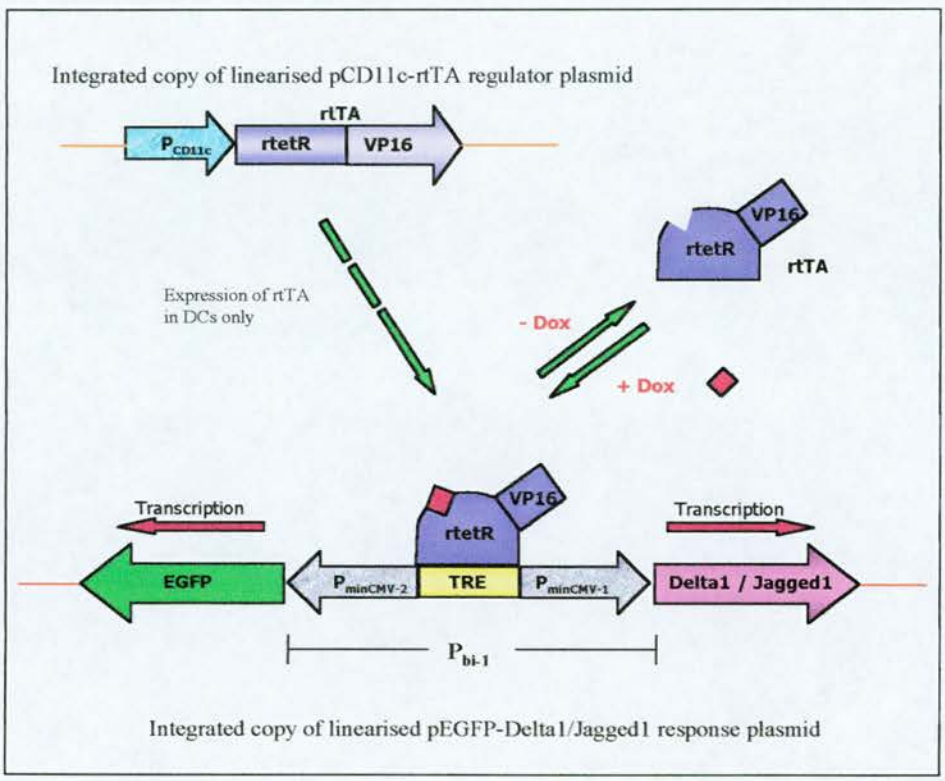


Figure VI.1. Schematic outline of the *tet*-regulated bidirectional transcription system.
Four amino acid exchanges in the repressor of the Tn10 Tc-resistance operon of *Escherichia coli* convey a reverse phenotype to the repressor (r*tet*R). The fusion protein (rtTA) composed of r*tet*R and a C-terminal portion of protein 16 of herpes simplex virus requires dox for binding to the Tc-responsive element (TRE). TRE consists of seven copies of the Tc-operator sequence (*tet*O) and is between two identical minimal human cytomegalovirus (CMV) promoters (P_{minCMV}), which form together the bidirectional promoter P_{bi-1}. P_{minCMV} lacks the enhancer that is part of the complete CMV promoter. Consequently, P_{bi-1} is silent in the absence of binding of rtTA to the TRE. $P_{minCMV-1}$ controls the expression of the gene of interest (Delta1 or Jagged1), and $P_{minCMV-2}$ controls the expression of the enhanced green fluorescent protein (EGFP).

VI.2 Generation of constructs for regulated DC-specific overexpression of Delta1 and Jagged1

VI.2.1 Generation of the pCD11c-rtTA vector

One line of mice was generated to express the rtTA under the control of the CD11c promoter, which limits expression to DCs (Fig. VI.1). A schematic outline of the cloning procedure is shown in figure VI.2. The rabbit β-Globin cDNA expression cassette provided the transgene with an intron and a polyadenylation signal (Kouskoff *et al.*, 1993). It has been demonstrated that efficient expression of a transgene requires splicing and polyadenylation of the transcribed product (te Riele *et al.*, 1992; Wurst and Joyner, 1993). Thus when a high level of expression is desired an intron downstream of the transcription start site, as well as a polyadenylation signal at its 3' end should be included. Here the β-Globin polyadenylation signal was replaced by the SV40 polyadenylation signal included in the rtTA DNA fragment.

The pBS-CD11c vector (Brocker *et al.*, 1997) and the pTet-On (Clontech Laboratories) were digested with *Cla*I or *Pvu*II, respectively and the ends were blunted. After a second digest of both constructs with *Eco*RI, a 8.9kb fragment of the pBS-CD11c vector containing the CD11c promoter and a 1.6kb fragment consisting of the rtTA and the SV40 polyadenylation signal, were isolated by gel extraction. The pBS-CD11c fragment was dephosphorylated to prevent religation. The rtTA fragment was cloned into the pBS-CD11c fragment producing the pCD11c-rtTA vector. Transformation was done in *E. coli* XL1-Blue MRF' competent cells (Stratagene). Six colonies were picked and plasmid DNA was isolated.

Clones were tested by restriction digest with *Xba*I and *Apa*I (Fig. VI.3). Expected lengths of resulting fragments are listed in table VI.1. *Xba*I digestion of plasmids with cloned rtTA fragment resulted in two fragments since both the opened pBI-CD11c and the rtTA contained a single *Xba*I restriction site (Fig. VI.3A). All six clones exhibited a 5.9 and a 4.6kb fragment confirming the correct orientation of the insert. Numbers of expected fragments with *Apa*I digest was not known since it was designated as a non-unique site on the restriction map of pBI-CD11c by T. Brocker (unpublished data). *Apa*I digest of the original pBI-CD11c vector (Ctrl) resulted in three fragments suggesting a third *Apa*I restriction site at unknown location.

Consequently the fragments had a length of 8.9-X, X and 0.6kb. All six clones digested with *Apa*I displayed the expected fragments confirming successful insertion of rtTA (Fig. VI.3B). In conclusion, 100% of the picked colonies had inserted the rtTA fragment in the correct orientation. The DNA sample of clone #6 was sent to DNASHEF Technologies for sequencing at the 5' and 3' cloning junctions for further confirmation.

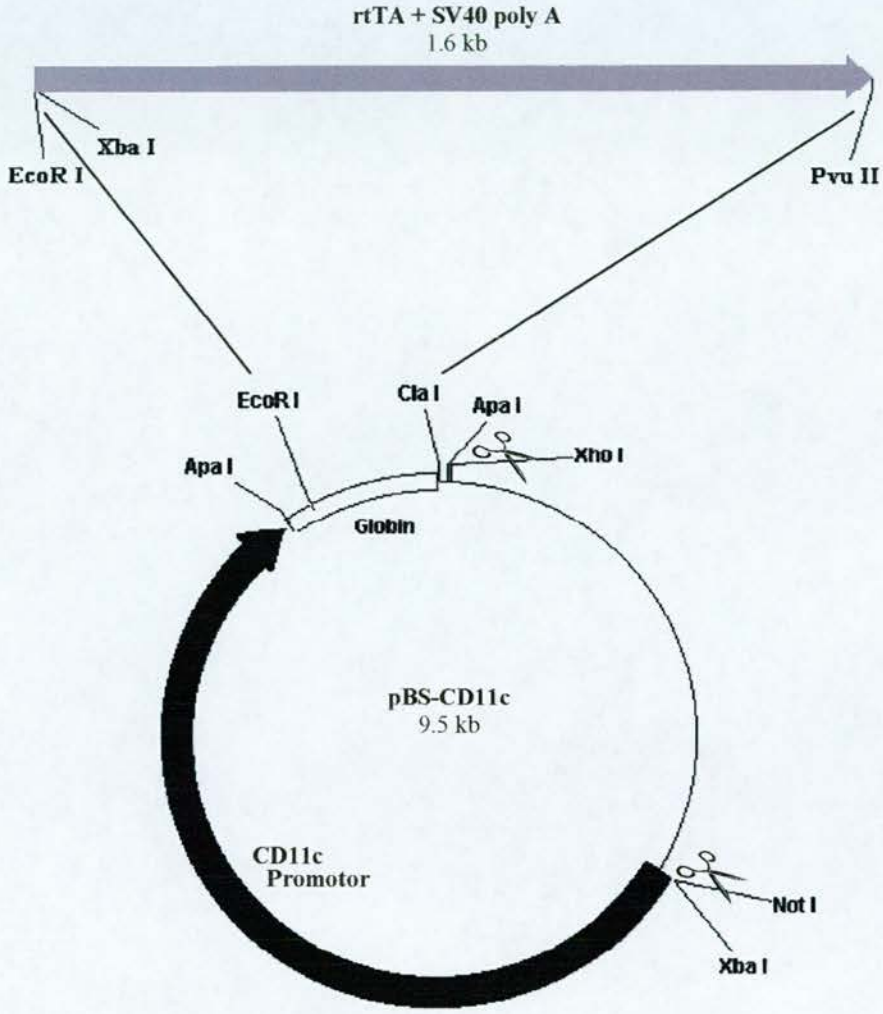


Figure VI.2. Schematic outline for the generation of the pCD11c-rtTA vector.
The rtTA fragment containing the SV40 polyadenylation signal (rtTA + SV40 poly A) was generated by digesting the pTet-On plasmid (Clontech Laboratories) with *Eco*RI and *Pvu*II (blunt). It was subsequently cloned into the *Eco*RI-*Cla*I (blunt) opened pBS-CD11c vector (Brocker *et al.*, 1997). The rabbit β-Globin cDNA expression cassette containing an intron and a polyadenylation signal (Globin) was partly replaced by the rtTA fragment. Restriction sites used for linearisation of the end product are indicated by scissors.

Table VI.1. Expected fragments after restriction digest of pCD11c-rtTA with *Xba*I and *Apa*I.

Restriction enzyme	Insert in the right orientation	Insert in the wrong orientation	No insertion	pBI-CD11c (Ctrl)
*Xba*I	5.9 kb 4.6 kb	7.5 kb 3.0 kb	8.9 kb	9.5 kb
*Apa*I	8.9 kb – X kb 1.6 kb X kb	8.9 kb – X kb 1.6 kb X kb	8.9 kb – X kb X kb	8.9 kb – X kb X kb 0.6 kb

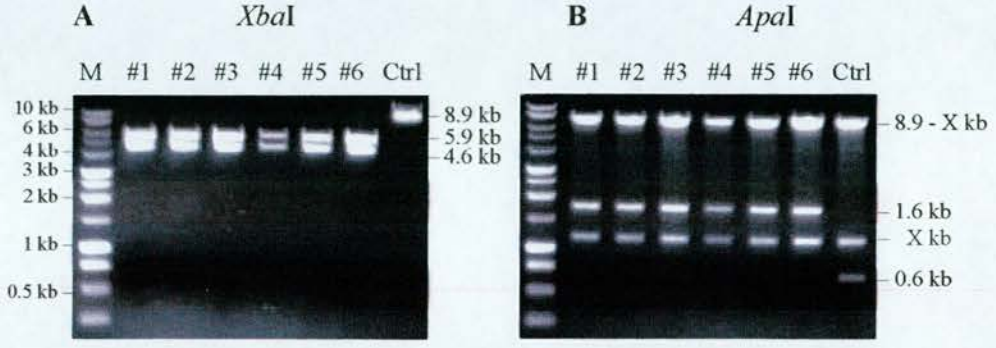


Figure VI.3. Restriction digest of pCD11c-rtTA vector.
Six different pCD11c-rtTA clones (#1-6) and the original pBS-CD11c vector as a control (Ctrl) were digested with *Xba*I (A) and *Apa*I (B) as described in materials & methods, section II.2.1. The digested products were run in a 0.8% agarose gel. M = 1kb DNA ladder

VI.2.2 Generation of the pEGFP-Jagged1 and pEGFP-Delta1 constructs

It was planned to generate two lines of mice expressing either murine Delta1 (Bettenhausen *et al.*, 1995) or human Jagged1 (Li *et al.*, 1998; Oda *et al.*, 1997) together with EGFP under the control of the P_{bi-1} (Fig. VI.1). Murine Jagged1 had not been completely sequenced or cloned at that time. Instead, the human homologue was used, which shows an overall amino acid identity with murine Jagged1 of 96% (Shimizu *et al.*, 1999) and is able to bind to murine Notch receptors (K. Tan, unpublished data; Hoyne *et al.*, 2000). Murine Delta1 and human

Jagged1 sequences (accession numbers X80903 and AF028593, respectively) were kind gifts of D. Ish-Horowicz and I. Le Roux. A schematic outline of the cloning procedure is shown in figure VI.4.

The pBI-L-Delta1 and pBI-L-Jagged1 constructs (K. Tan, unpublished data) were double digested with *Mlu*I and *Eco*RV resulting in a 2.1 and a 3.6kb fragment of murine Delta1 and human Jagged1, respectively. The pBI-EGFP vector (Clontech Laboratories) was linearised with *Nhe*I and the ends were blunted. A second digest with *Mlu*I gave rise to a 5.1kb fragment with one cohesive and one blunt end, which were then dephosphorylated to prevent religation. The Delta1, Jagged1 and the linearised pBI-EGFP fragments were purified by gel extraction. Delta1 and Jagged1 were ligated into the pBI-EGFP vector and transformed into *E. coli* XL1-Blue MRF' competent cells. Six colonies of each vector were picked and plasmid DNA was isolated.

Clones were digested with *Bam*HI or *Not*I for pEGFP-Delta1 and *Bam*HI or *Sac*I for pEGFP-Jagged1 to check for insertion and correct orientation of the gene of interest (Fig. VI.5). Expected lengths of resulting fragments are listed in table VI.2. Five out of six pEGFP-Delta1 plasmids contained the Delta1 insert in the correct orientation as demonstrated by restriction digest with *Bam*HI and *Not*I (Fig. VI.5 A&B). Restriction digest of pEGFP-Jagged1 with *Bam*HI gave rise to the three expected fragments of 0.6, 0.8 and 7.4kb in five out of six constructs confirming insertion of Jagged1. *Sac*I digestion resulted in fragments of 0.3, 3.2 and 5.0kb. The expected 0.1 and 0.05kb fragments were too small to be visible on the gel. An additional fragment marked with an arrow is visible between 8 and 10kb, which is most probably the undigested pEGFP-Jagged1 vector (8.8kb) due to partial digestion of the vector. It therefore can be concluded that the Jagged1 fragment was inserted in the correct orientation in five of the six plasmids.

In summary, 83% of the picked colonies of the pEGFP-Delta1 and pEGFP-Jagged1 had inserted the Delta1 and Jagged1 fragments, respectively, in the correct orientation. DNA samples of the pEGFP-Delta1 clone #6 and the pEGFP-Jagged clone #5 were sent to DNASHEF Technologies for sequencing at the 5' and 3' cloning junctions for further confirmation.

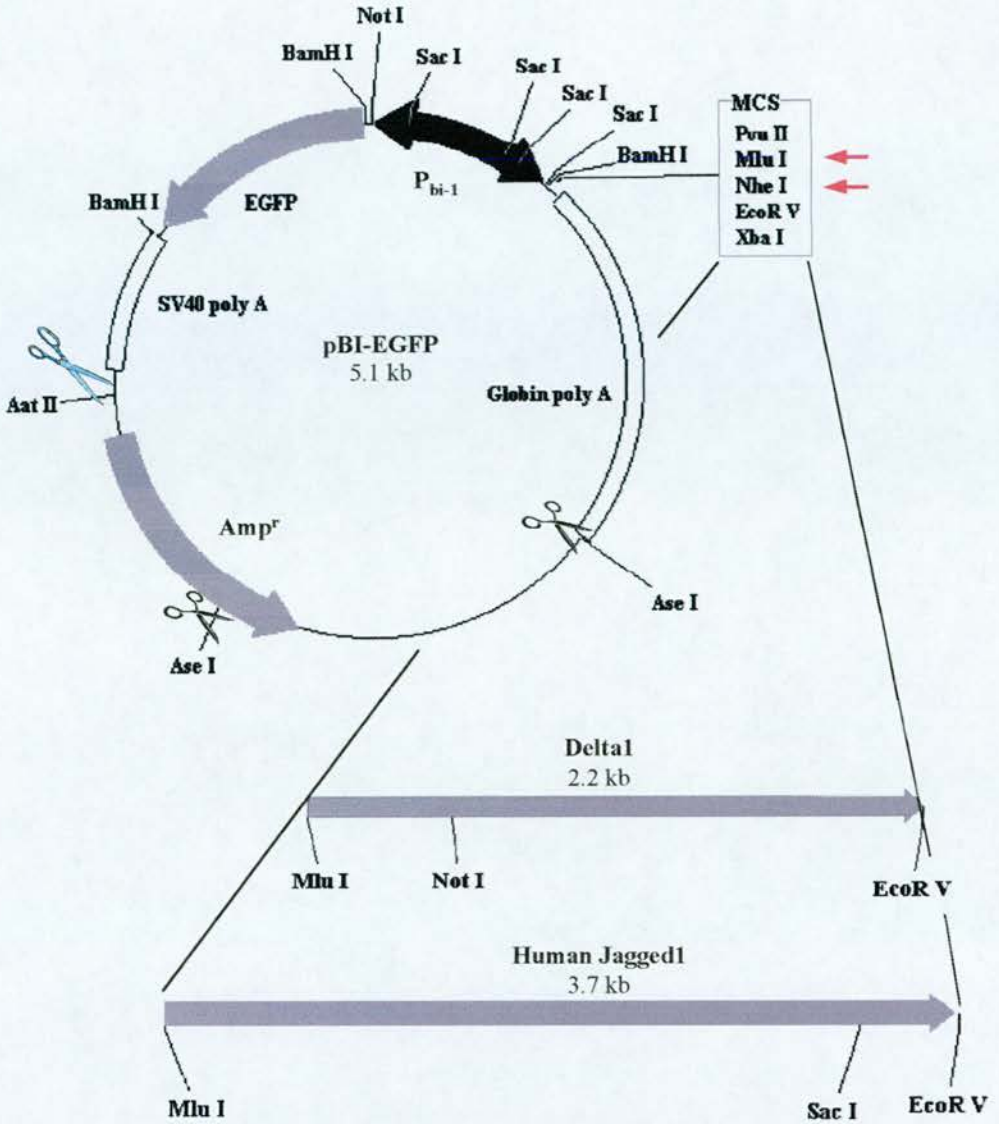


Figure VI.4. Schematic outline for the generation of the pEGFP-Delta1/Jagged1 vectors.
The Delta1 and Jagged1 fragments were generated by double digesting the pBI-L-Delta1 and pBI-L-Jagged1, respectively (K. Tan, unpublished data) with *Mlu*I and *Eco*RV. They were subsequently cloned into the *Mlu*I-*Nhe*I (blunt) opened pBI-EGFP vector (Clontech Laboratories) resulting in the pEGFP-Delta1 and pEGFP-Jagged1 constructs. Black and blue scissors indicate restriction sites used for linearisation of the pEGFP-Delta1 and pEGFP-Jagged1 constructs, respectively. Red arrows indicate restriction sites on pBI-EGFP used for cloning.
P_{bi-1}: bidirectional tetracycline-responsive promoter, Amp^r: Ampicillin resistance gene, EGFP: EGFP gene, SV40 poly A: fragment containing the SV40 polyadenylation signal, Globin poly A: fragment containing the β-globin polyadenylation signal, MCS: multiple cloning site.

Table VI.2. Expected fragments after restriction digest of pEGFP-Delta1/Jagged1.

Vector	Restriction enzyme	Insert in the right orientation	Insert in the wrong orientation	No insertion or pBS-EGFP (Ctrl)
pEGFP-Delta1	*Bam*HI	5.9 kb 0.8 kb 0.6 kb	5.9 kb 0.8 kb 0.6 kb	3.7 kb 0.8 kb 0.6 kb
	*Not*I	6.2 kb 1.1 kb	5.0 kb 2.3 kb	5.1 kb
pEGFP-Jagged1	*Bam*HI	7.4 kb 0.8 kb 0.6 kb	7.4 kb 0.8 kb 0.6 kb	3.7 kb 0.8 kb 0.6 kb
	*Sac*I	5.0 kb 3.2 kb 0.3 kb 0.1 kb 0.05 kb	7.8 kb 0.5 kb 0.3 kb 0.1 kb 0.05 kb	4.7 kb 0.3 kb 0.1 kb 0.05 kb

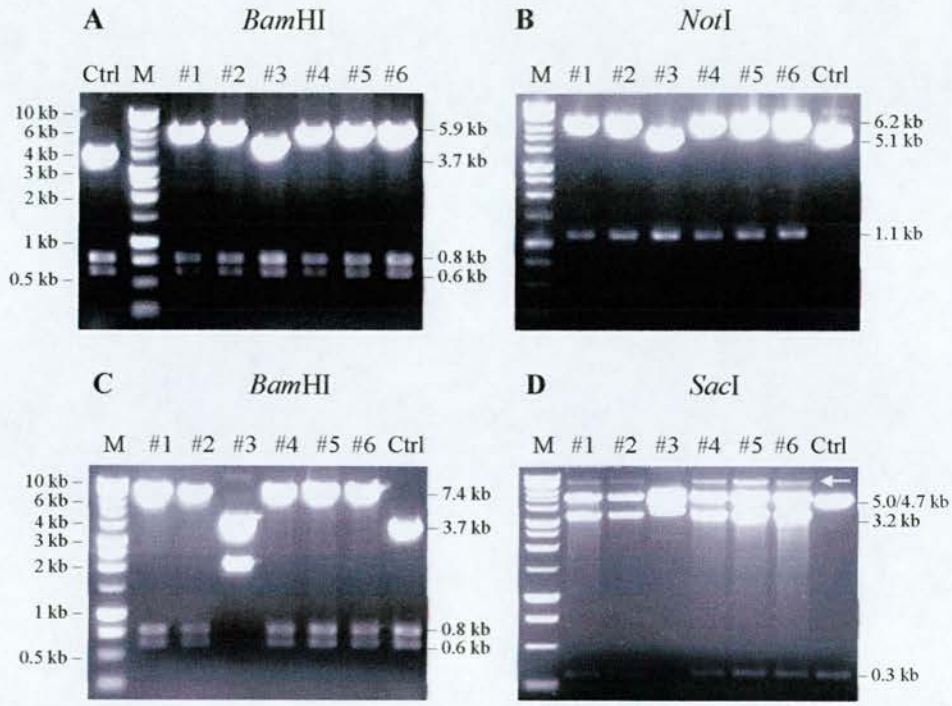


Figure VI.5. Restriction digest of pEGFP-Delta1/Jagged1 vectors.
Six different pEGFP-Delta1 (#1-6, A and B) and pEGFP-Jagged1 clones (#1-6, C and D) were digested with *Bam*HI (A) or *Not*I (B) and *Bam*HI (C) or *Sac*I (D), respectively, as described in materials & methods, section II.2.1. The original pBI-EGFP vector was included in each restriction digest as a control (Ctrl). The digested products were run in a 0.8% agarose gel. The band marked with a white arrow is most probably undigested pEGFP-Jagged1 vector. M = 1kb DNA ladder

VI.2.3 Upscaling and preparation of the constructs for microinjection

The three constructs, pCD11c-rtTA, pEGFP-Delta1 and pEGFP-Jagged1, were upscaled for linearisation by doing maxipreps. The pCD11c-rtTA construct was double digested with *Not*I and *Xho*I, whereas as pEGFP-Delta1 and pEGFP-Jagged1 were single digested with *Ase*I and *Aat*II, respectively. Expected fragments are shown in table VI.3. Linearised fragments were purified by gel extraction, ethanol precipitated and quantified by running on a gel together with quantification standards (Fig. VI.6)

Table VI.3. Expected fragments after linearisation of pCD11c-rtTA, pEGFP-Delta1 and pEGFP-Jagged1.

Construct	Restriction enzyme	Undigested construct	Digested construct *
pCD11c-rtTA	*Not*I *Xho*I	10.5 kb	**7.5 kb** 3.0 kb
pEGFP-Delta1	*Ase*I	7.3 kb	**6.1 kb** 1.2 kb
pEGFP-Jagged1	*Aat*II	8.8 kb	**8.8 kb**

* Fragment used for microinjection is indicated by bold letters

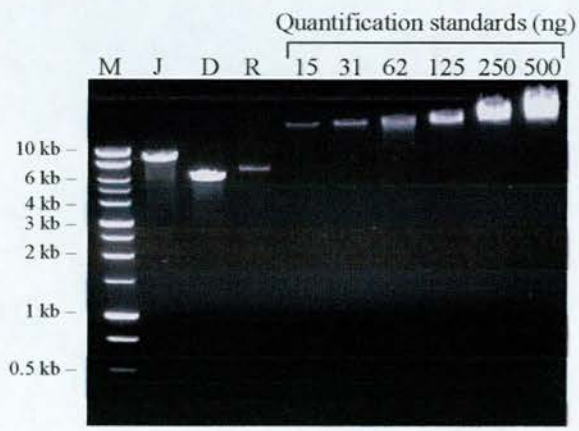


Figure VI.6. Quantification of linearised fragments.
Linearised fragments of pEGFP-Jagged1 (J), pEGFP-Delta1 (D) and pCD11c-rtTA (R) were run in a 0.8% agarose gel together with quantification standards (15, 31, 62, 125, 250 and 500ng). M = 1kb DNA ladder

Pronuclear microinjection of the DNA fragments was performed on murine fertilised zygotes from hormonally superovulated females of the F1 generation of C57BL/6J x CBA/CA ($H\text{-}2^{b/k}$) mated with males of the same genotype at the Babraham Institute, Cambridge. The use of this strain of mice is known to yield a high number of high-quality oocytes, which are relatively resistant to *in vitro* manipulations (Jackson and Abbott, 2000).

VI.3 Screening and breeding of the founder mice

With linear DNA up to 30% of successfully injected and transferred embryos can be transgenic (Jackson and Abbott, 2000). However, the average lies between 10 and 20% (J. Saunders, personal communication).

I received 10, 27 and 24 founder mice of the CD11c-rtTA, EGFP-Jagged1 and EGFP-Delta1 line, respectively. The founders were screened for incorporation of the microinjected genes by PCR. Genomic DNA was isolated from tail tips and analysed by PCR (Fig. VI.7). 6 out of 27 (22%) and 7 out of 24 (29%) of the EGFP-Jagged1 and EGFP-Delta1 founders were positive for the EGFP transgene (Fig. VI.7 A&B). However, none of the CD11c-rtTA founders contained the transgene (Fig. VI.7C). To exclude the possibility that the DNA was lost during the extraction process, a PCR amplifying the β-Actin gene was carried out. As demonstrated in figure VI.7D genomic DNA was present in all samples. Therefore, it was concluded that none of CD11c-rtTA founders were positive for the transgene.

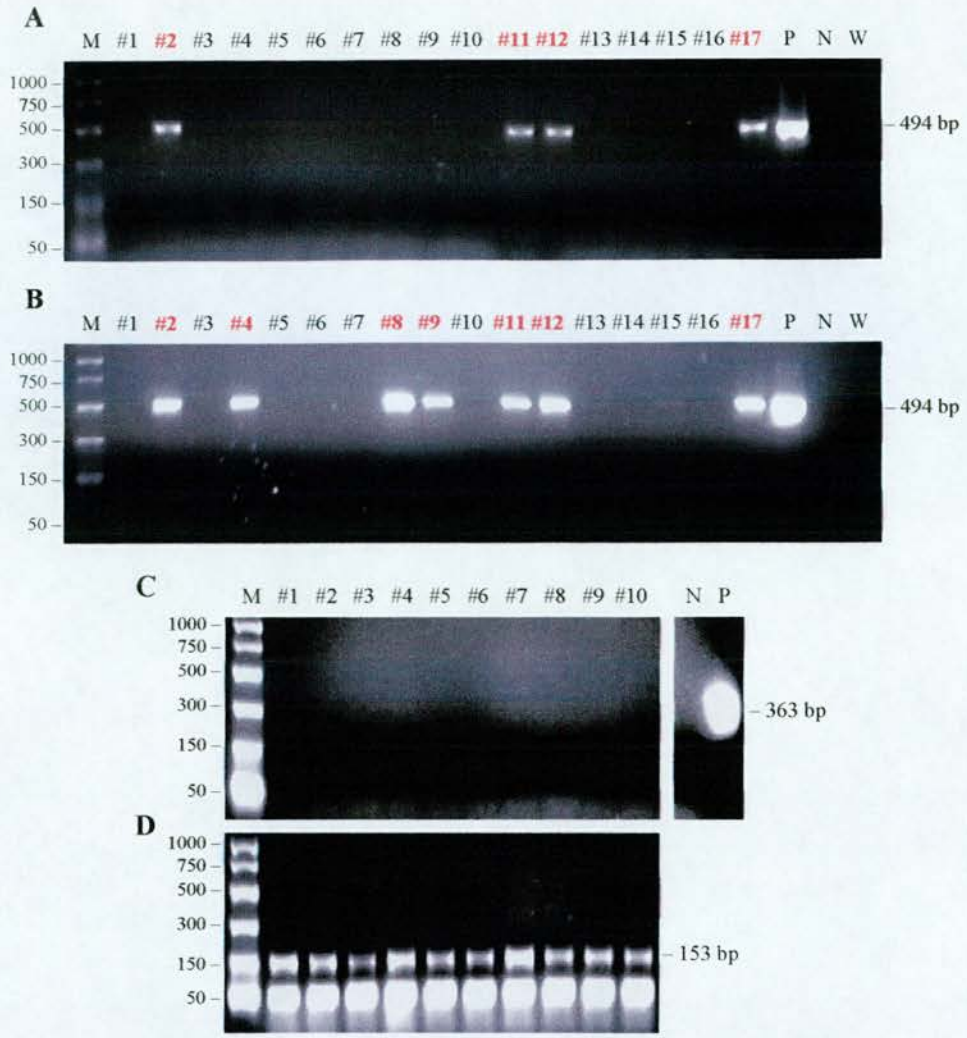


Figure VI.7. Screening of the founders.
Genomic DNA was extracted from tail tips of the founder.
[A & B] The transgene EGFP was amplified by PCR from DNA of the mouse lines EGFP-Jagged1 and EGFP-Delta1 and the products run in a 2% agarose gel. Founders #1-17 of the EGFP-Jagged1 and EGFP-Delta1 lines [A and B, respectively] are shown.
[C & D] The transgene rtTA [C] and the endogenous gene for β-Actin [D] were amplified by PCR from DNA of the CD11c-rtTA line and the products were run in a 2% agarose gel.
Founders positive for the transgene are indicated in bold numbers. M = PCR marker, N = Negative control (DNA of a wild-type mouse), P = Positive control (plasmid DNA of the corresponding gene), W = Water control

It was decided to breed three separate lines originating from three different founders for both of the transgenic lines, EGFP-Jagged1 and EGFP-Delta1, thereby increasing the probability of obtaining functional transgenic mice. This strategy was necessary for several reasons: a) In approximately 10-30% of transgenic animals integration of the transgene occurs not

during the on-cell stage but later in development (Jackson and Abbott, 2000). The founder mouse will then be mosaic for the transgene and germline transmission is not guaranteed. b) A possibility exists that the random integration of the transgene may have knocked out an endogenous gene. Many genes do not show an obvious phenotype when heterozygously mutated. When such mice are crossed to achieve homozygosity, the resultant transgenic line may show a phenotype caused by disruption of the genetic locus (Kilby *et al.*, 1993). c) Relatively rare are integrations at several sites in the mouse genome. In these cases, the offspring of the transgenic founder are likely to carry only a subset of the integrated copies in the founder animal, due to segregation of the chromosomes. d) Integration of 1-50 copies of transgene is normal, but integration of up to 1000 copies has been observed (Doetschman *et al.*, 1988). The individual copies are usually found in a tandem array as head-to-tail fusions. e) Additionally to the dependence on the number of copies of the transgene, the level of expression is often influenced by the genomic sequences flanking the site at which the transgene is integrated. Flanking sequences may contain regulatory elements of nearby genes that act on the promoter of the transgene as an enhancer or as a repressor.

To identify founders with high copy numbers of transgenes, a PCR was carried out amplifying the transgenes EGFP and Jagged1 or Delta1 together with β-Actin, which was used for normalisation (Fig. VI.8). As expected, all EGFP-Jagged1 founders with incorporated EGFP transgene were also positive for the transgene human Jagged1, whereas the wild-type control did not contain either of the transgenes (Fig. VI.8A). The primers used for Delta1 were found to be intron-spanning because they amplified a product at a higher molecular weight then expected by running a computer-based PCR programme (DNA Strider) using the mRNA-derived sequence of Delta1 (Fig. VI.8B). The programme predicted a PCR product of 571 base pairs (bp), which was obtained with the plasmid control and the EGFP-positive founders. The length of the intron-spanning PCR product cannot be predicted because the genomic sequence of the murine Delta1 gene has not yet been completely identified. The level of Jagged1 or Delta1 transgene seems to be roughly proportional to the amount of EGFP transgene. For example EGFP-Jagged1 founder #26 contained relatively high levels of both transgenes, whereas founder #11 exhibited weak bands for both transgenes. This was expected because the two transgenes were located on the same DNA fragment. Three transgenic founders of both lines (EGFP-Jagged1 #17, #19 and #26 and EGFP-Delta1 #8, #12 and #17) were chosen for breeding. By picking mice exhibiting strong bands of transgenes and weak

bands of β-Actin it was hoped to increase the probability of obtaining mouse lines with high expression of transgenes. Each of these founders was backcrossed onto the C57BL/6 background (H-2^b) for several generations giving rise to a transgenic line, e.g. the EGFP-Jagged1 #17 line, with a H-2^b haplotype.

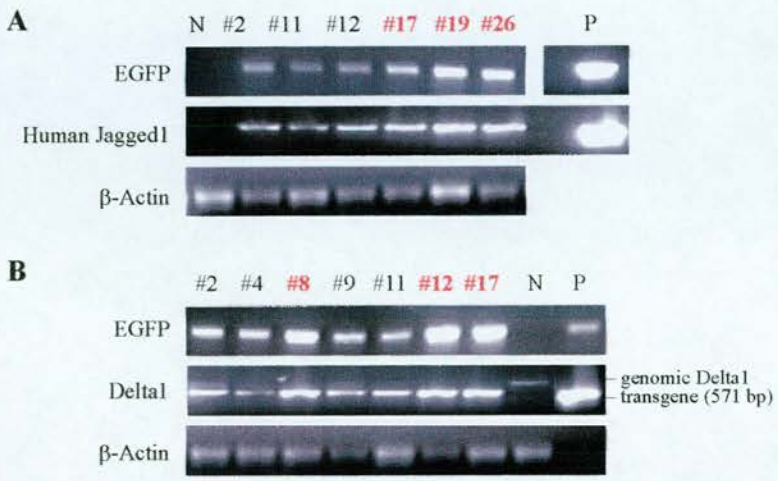


Figure VI.8. Comparison of levels of transgene copies.
[A] EGFP, human Jagged1 and β-Actin were amplified by PCR from genomic DNA of the transgenic founders of the EGFP-Jagged1 line.
[B] EGFP, Delta1 and β-Actin were amplified by PCR from genomic DNA of the transgenic founders of the EGFP-Delta1 line.
The products were run in a 2% agarose gel. N = Negative control (DNA of a wild-type mouse), P = Positive control (plasmid DNA of the corresponding gene)

VI.4 Discussion and Outlook

With 22 and 29% of transgenic EGFP-Jagged1 and EGFP-Delta1 founders, respectively, successful generation of transgenic mice were above average (J. Saunders, personal communication). Only 10 founders of the CD11c-rtTA line were received. Even in the range of the expected average, only one or two transgenic mice were expected. It was unfortunate that none of the founders contained the transgene. However, collaboration with M. Dallman at Imperial College of Science, Technology and Medicine, London, was started. They were also

generating the CD11c-rtTA mouse line in order to produce DC-specific inducible knock-out mice using the Cre-loxP recombinase system (reviewed in Kilby *et al.*, 1993).

First, transgenic mice will be bred onto the C57BL/6 background (H-2^b). This will allow using the Der p 1 system to investigating the role of Notch ligands in DCs during tolerance induction. All three lines, the regulator mice (CD11c-rtTA) and the responder mice (EGFP-Jagged1/Delta1) will have to be crossed to obtain homozygous transgenic lines. A homozygous regulator mouse can then be mated with a homozygous responder mouse producing offspring that are heterozygous for all three transgenes, rtTA, EGFP and Jagged1 or Delta1 (referred to as CD11c-Jagged1 or CD11c-Delta1, respectively).

The CD11c-Jagged1/Delta1 mice will be examined for specificity and leakiness of transgene expression. In theory, the rtTA should only be expressed in CD11c-positive cells. Brocker *et al.* reported that transgenes under the control of the CD11c promoter were specifically expressed in DCs (Brocker *et al.*, 1997) even though other cell types can also express the CD11c integrin (Hogg *et al.*, 1986; Myones *et al.*, 1988). Leakiness in the *tet* system can arise in two ways. Firstly, the rtTA protein may bind to the P_{bi-1} promoter in the absence of dox and induce weak transgene expression. Secondly, the P_{bi-1} promoter itself may be slightly leaky enabling low level of transcription in the absence of rtTA. To investigate these issues, CD11c-positive and -negative cells can be isolated from spleens of CD11c-Jagged1/Delta1 mice and total RNA extracted. Only CD11c-positive cells should express the rtTA transgene, whereas none of the cell types should contain EGFP transcripts. The same experiment should be conducted with mice that have received dox in their drinking water starting at several time points prior to being sacrificed. Kirstner *et al.* demonstrated that 24 hours of dox administration is sufficient to induce maximal transcription of the rtTA-activated transgene (Kistner *et al.*, 1996). CD11c-positive cells of dox-treated mice should express increasingly high levels of transgene proportional to the length of dox administration ending in a plateau where saturation was reached. In contrast, CD11c-negative should remain EGFP-negative.

After examining the specificity and leakiness of CD11c-Jagged1/Delta1 mice receiving dox in their drinking water, the next step would be to characterise their phenotype. Cells of spleen, lymph nodes and bone marrow would be double stained for CD11c and other cell surface proteins such as activation markers and costimulatory molecules and analysed by flow cytometry. EGFP expression can be measured with the FL1 channel (Cormack *et al.*, 1996). $CD11c^+$ cells should also be EGFP-positive. In addition to characterising their phenotype the number of $CD11c^+$ DCs and other cell types will be examined. Notch activation has been

shown to induce apoptosis in B cells and monocytes (Morimura *et al.*, 2000; Ohishi *et al.*, 2000), whereas it protected thymocytes and haematopoietic precursors from apoptosis (Deftos *et al.*, 1998; Han *et al.*, 2000). Therefore, alterations in the proportion or in the total number of cells might be expected.

The *tet* system can also be applied to *in vitro* analysis (Gossen and Bujard, 1992). Growing DCs derived from bone marrow of CD11c-Jagged1 in the presence of dox *in vitro* may answer the question whether or not the observed upregulation of Jagged1 during maturation of DCs is directly linked to that process. If this is the case, then the induction of Jagged1 by dox may induce maturation, alter the expression of maturation markers and/or stimulate cytokine production. Furthermore, it will be of interest to investigate the effect of Jagged1 or Delta1 overexpression in DCs on T cell activation in MLRs. The cytokine profile, especially the secretion of IFNγ, of the activated T cells would be studied. A similar analysis could be applied in order to determine the function of Delta1 in DCs as regards its ability to regulate their function and maturation.

Experiments involving the transfer of DCs overexpressing Jagged1 to wild-type mice will be of special interest because this may have implications in treating allergy, autoimmune diseases or in prolonging transplant survival.

An experimental mouse model to study the allergic response to house dust mite (HDM) was developed in our group (Hawrylowicz *et al.*, 1995; Hoyne *et al.*, 1993a). Mice of the H-2^b haplotype (C57BL/6) are high responders to the HDM protein Der p 1 and recognise four distinct $CD4^+$ T cell epitopes on the protein (Hoyne *et al.*, 1993b). Systemic administration of the immunodominant peptide of Der p 1 in adjuvant induced productive immunity, whereas the same peptide when delivered intranasally (i.n.) promoted hyporesponsiveness to all epitopes of Der p 1, a process called linked suppression (Hoyne *et al.*, 1993c). This mechanism is similar to bystander suppression observed in oral tolerance (Miller *et al.*, 1991), in that both involve the generation of regulatory T cells. The injection of Jagged1 overexpressing APCs pulsed with the immunodominant peptide of Der p 1 rendered mice unresponsive to an immunogenic challenge with the intact Der p 1 protein (Hoyne *et al.*, 2000). Furthermore, regulatory $CD4^+$ T cells were induced which could transfer antigen-specific tolerance to naïve mice similar to the regulatory T cells induced by i.n. delivery of the immunodominant Der p 1 peptide. Repeating these experiments with BM-derived DCs overexpressing Jagged1 and pulsed with Der p 1 may induce regulatory T cells in a similar

fashion to Jagged1$^+$ APCs, which may have implications in treating atopic persons with allergic reactions.

Most cases of organ-specific autoimmune diseases develop as a consequence of self-reactive CD4$^+$ T cells. The T_H1/T_H2 balance can have an impact on whether autoimmunity develops or not (reviewed Charlton and Lafferty, 1995). T_H1 cells have been implicated in the development of autoimmunity, whereas T_H2 cells not only protect against the induction of disease but also against progression of established disease. Injecting DCs overexpressing Jagged1 and pulsed with autoantigen may alter the T_H1/T_H2 balance by inhibiting T cells to produce IFNγ similar to the T cell response to Jagged1$^+$ L cells (Chapter V) or induce regulatory CD4$^+$ T cells (Hoyne *et al.*, 2000).

It was proposed that DCs of the allograft were responsible for immunogenicity (reviewed in Steinman, 1991). Donor DCs are potent stimulators of the direct pathway of allorecognition, in which recipient T cells respond to peptides presented on donor MHC products or to the donor MHC molecules themselves. Reduced DC function in the direct pathway was used to explain the acceptance of certain allografts that have been depleted of passenger leukocytes (Bowen *et al.*, 1980; Talmage *et al.*, 1976). However, depletion of donor APCs prevented the induction of tolerance to heart and liver transplants (Josien *et al.*, 1998; Sun *et al.*, 1995). Furthermore, in some cases tolerance was re-established if donor-type DCs were cotransferred with APC-depleted grafts (Josien *et al.*, 1998). Thus, DCs are capable to induce both, immunogenicity or tolerance to transplants. The use of genetically engineered DCs to pre-treat the donor allows the manipulation of the immune response towards tolerance rather than immunogenicity. Genetically engineered DCs that for example express IL-10 (Takayama *et al.*, 1998), TGF-β (Lee *et al.*, 1998), FasL (Min *et al.*, 2000) or CTLA-4-Ig (O'Rourke *et al.*, 2000) can induce alloantigen-specific T cell hyporesponsiveness and enhance the survival of allografts. It will be of interest to investigate whether pre-treatment of donor animals with Jagged1 overexpressed in donor (direct pathway) and recipient DCs pulsed with donor-peptides (indirect pathway) will also prolong graft survival.

As described above, DCs genetically engineered to overexpress Jagged1 may have important therapeutic implications. Human DCs can be propagated from peripheral blood mononuclear cells, CD34$^+$ progenitors and cord blood (Caux *et al.*, 1996; Romani *et al.*, 1996; Sallusto and

Lanzavecchia, 1994) and overexpression of Jagged1 could be achieved by retroviral-mediated gene transfer (Hoyne *et al.*, 2000). However, viral-mediated transfections are not popular because of the risks they bring along by administering cells containing viral DNA to patients. Several strategies for non-viral gene transfer such as electroporation, gene gun and liposome-based techniques exist which could be applied to allow the treatment of patients with Jagged1 transfected DCs.

VII Conclusions

Notch and DCs

DC maturation is important for their function as professional APCs because it changes their chemokine pattern, which allows them to migrate to T cell areas in secondary lymphoid organs, upregulates adhesion molecules necessary for a stable DC-T cell interaction and increases the expression of MHC/peptide complexes and costimulatory molecules crucial for the stimulation of T lymphocytes. Work presented in this thesis (chapter IV) showed that maturation stimuli such as TNFα or LPS induced expression of Jagged1 in DCs and differentially regulated the expression of Delta1. The profile of Notch ligands on DCs depended not only on the maturation state but also on the microenvironment, as shown by the differential expression of Jagged1 if the maturation occurred in the presence of IL-10. Therefore, the Notch ligands may add to the information brought by the DCs from the periphery to the secondary lymphoid organs thereby contributing to the "signal 3" responsible for the modulation of the immune response.

It has been proposed that Notch signalling may regulate the destabilisation of the actin cytoskeleton, which is important for cell migration, although direct evidence is still missing (Dye *et al.*, 1998; Small *et al.*, 2001; Zecchini *et al.*, 1999). More important, Notch is known to regulate differentiation of many cells including lymphocytes (see section I.4). Therefore, it is of interest to investigate whether Notch ligands displayed by APCs such as DCs modulate the differentiation of naïve T lymphocytes upon the initial encounter.

Notch and T lymphocytes

In chapter III, I have shown that T cells express crucial downstream components of the Notch signalling pathway, in addition to several Notch receptors. However, upon culturing *in vitro* without stimulation, transcripts of targets of Notch signalling such as Hes1 or Deltex1 decline rapidly and the expression of Hes1 can only be partially restored by activation with anti-CD3/anti-CD28 or PMA/ionomycin. This suggests a requirement for Notch activation by ligands expressed on accessory cells such as APCs. Indeed, coculturing of T lymphocytes with

transfected L cells expressing Jagged1 or Delta1 induces strong activation of Hes1 transcription demonstrating that peripheral T lymphocytes are able to receive Notch signalling presumably by interacting with ligand expressing APCs. In contrast, Hes5 expression was not affected by Jagged1 or Delta1 expressed on L cells. However, activation of $CD4^+$ T lymphocytes in the presence of IL-10 upregulated Hes5 transcripts. It has been shown by others that Hes1 transcription was not affected in CBF mutants suggesting the existence of a CBF-independent pathway, which may not involve the Notch receptors (de la Pompa *et al.*, 1997). Hence, triggering of the TCR/CD28 signalling pathways in the presence of IL-10 could be responsible for the increased expression of Hes5. Alternatively, Notch activation by ligands expressed on neighbouring T cells cannot be excluded as the cause for the induction Hes5 in the presence of IL-10.

Notch and cellular expansion/apoptosis

Many groups have focused on Notch signalling in thymocytes. It was suggested that Notch1 activation protects from glucocorticoid-induced or TCR-mediated apoptosis (Deftos *et al.*, 1998; Jehn *et al.*, 1999). However, in my system using peripheral T lymphocytes (chapter V), no change of cell survival or apoptosis was observed consistent with data published by Wolfer *et al.* (2001). Furthermore, proliferation of $CD4^+$ and $CD8^+$ T cells in response to allogeneic L cells was not altered in response to Jagged1, and only slightly increased in response to Delta1, whereas Allman and colleagues reported a strong increase in expansion of thymocytes upon Notch activation in the presence of TCR signalling leading to T cell leukaemia (Allman *et al.*, 2001). These differences may be explained by either the distinct differentiation state, thymocytes vs. mature T lymphocytes, or by the possible activation of distinct Notch receptors. Whereas most of the studies on Notch signalling in thymocytes were concentrated on Notch1, it remains unclear which Notch receptor(s) led to the upregulation of Hes1 in T cells activated by ligand expressing allogeneic L cells.

Notch and regulatory T cells

As shown in chapter V, Jagged1-induced Notch activation in T cells decreased secretion of IFNγ during a MLR. Interestingly, Delta1-mediated Notch activation did not alter IFNγ

secretion, even though both ligands induced the transcription of Hes1. Both Jagged1 and Delta1 are able to bind to several Notch receptors albeit with different affinities (Shimizu *et al.*, 2000; Shimizu *et al.*, 1999). However, it is not clear whether they also activate all the Notch receptors they bind to. This may explain the differences of Jagged1 vs. Delta1 induced Notch activation. Furthermore, Jagged1 upregulates Hes1 transcripts at a higher extent than Delta1.

Hoyne and colleagues reported a role for Notch signalling in the induction of $CD4^+$ T_{reg} cells *in vivo* (Hoyne *et al.*, 2000). Naïve mice were injected with Jagged1 expressing APCs pulsed with the immunodominant peptide of Der p 1 prior to immunogenic challenge with the whole protein. $CD4^+$ T cells of these mice were hyporesponsive *in vitro* when restimulated with peptide and could transfer tolerance to naïve mice. Interestingly, these T cells were unable to secrete IFNγ consistent with my data showing that Jagged1 decreased secretion of IFNγ by T cells stimulated *in vitro*. Furthermore, $CD4^+CD25^+$ T_{reg} cells, which are known to produce little IFNγ (Jonuleit *et al.*, 2001), express high levels of Hes1 upon activation reinsuring a possible link between Hes1 upregulation and decreased secretion of IFNγ (Ng *et al.*, 2001). Although these two T_{reg} population have different origins, $CD4^+$ T_{reg} cells in Hoyne's model were induced in the periphery (Hoyne *et al.*, 1993c), whereas $CD4^+CD25^+$ T_{reg} cells are thymus-derived (Itoh *et al.*, 1999), both suppress naïve T cells in a cytokine-independent but cell contact-dependent manner (Dieckmann *et al.*, 2001; Hoyne *et al.*, 1997; Thornton and Shevach, 1998). This poses the question whether Notch signalling may also control suppression of naïve T lymphocytes by T_{reg} cells. Interestingly, $CD4^+CD25^+$ T_{reg} cells, but not $CD4^+CD25^-$ T lymphocytes increased expression of Delta1 upon stimulation (Ng *et al.*, 2001). Furthermore, Delta1 transcripts were induced in the draining lymph nodes of mice tolerised by intranasal (i.n.) peptide delivery (Hoyne *et al.*, 1999). Adoptive transfer of Delta1 transfected $CD4^+$ T cells specific for the immunodominant peptide of Der p 1 inhibited responses to both the dominant and minor epitopes of Der p 1, a process termed 'linked suppression'. Thereby these $Delta1^+$ $CD4^+$ T cells mediate similar effects to T_{reg} cells induced by i.n. peptide administration or by injection of Jagged1 expressing DCs. The phenomenon of linked suppression remains unclear. It is possible that T_{reg} cells transmit an inhibitory signal to naïve T lymphocytes clustered around the same APC. Alternatively, T_{reg} cells may signal to the APC such that they develop a tolerogenic phenotype and inhibit the expansion of T lymphocytes reactive for the minor epitopes. Delta1 expressed by T_{reg} may well be a potential candidate for this inhibitory signal.

Notch and IFNγ transcription

The mechanism of Notch-induced downregulation of IFNγ secretion has not been explored yet. Notch activation interferes with several signalling pathways important for T cell activation and cytokine production, such as NF-κB or Ras/JNK signalling. There exist controversial reports about Notch regulating NF-κB. The activated intracellular domain of Notch1 has been shown to inhibit NF-κB activity similarly to IκB (Guan *et al.*, 1996; Wang *et al.*, 2001). On the other hand, CBF1 inhibits NF-κB transcription in the absence of Notch1 signalling (Oswald *et al.*, 1998; Palmieri *et al.*, 1999). Therefore, Notch signalling may positively or negatively regulate NF-κB-dependent cytokine production. Although Jagged1 expressed on L cells downregulated secretion of IFNγ by activated T cells as shown in chapter V, it did not seem to inhibit transcription of IFNγ. Thus, I hypothesise that Jagged1-induced Notch signalling may interfere with the secretion rather than transcription of IFNγ.

Notch and IFNγ secretion

Notch activation has been shown to interact with the regulation of the cytoskeleton in *Drosophila* (Buckles *et al.*, 2001; Dye *et al.*, 1998; Zecchini *et al.*, 1999). During dorsal closure of *Drosophila* development, Notch represses JNK independently of Su(H) (the *Drosophila* homologue of CBF) activity (Zecchini *et al.*, 1999). The authors speculate that this inhibition may happen at the level of Rac (a GTP-binding protein), which activates JNK signalling and modulates cytoskeletal activity (Harden *et al.*, 1995). Interestingly, Rac-1 constitutes a major pathway involved in NF-κB-mediated TNFα secretion following LPS challenge in macrophages (Sanlioglu *et al.*, 2001) and is activated by Vav upon TCR/CD28 stimulation in T lymphocytes (Song *et al.*, 1999a; Stinchcombe and Griffiths, 2001). Since Rac-1 is important for the secretion of mediators such as cytokines, Notch signalling may decrease IFNγ secretion by repressing Rac-1 activity in T cells.

It will be of interest to investigate in more detail the role of Notch signalling in the downregulation of IFNγ secretion. The analysis of intracellular IFNγ in T cells activated by Jagged1 expressing L cells in the absence of any secretion inhibitors such as Brefeldin A or monensin may shed light on this issue. If Notch activation interferes with secretion of IFNγ, I would expect to see increased amounts of IFNγ in T cells activated by Jagged1^{+} L cells

compared to Delta1^{+} or I-A^{b+} L cells. However, if Notch interferes with IFNγ secretion by repressing Rac-1 activity, differences in intracellular IFNγ will not been seen if PMA/ionomycin are used for the induction of cytokine production: Degranulation of mast cells expressing a dominant negative mutant of Rac-1 is rescued by stimulation with PMA/ionomycin suggesting the location of Rac-1 activity upstream of PKC and inositol trisphosphate (IP_3)-induced increase of cytoplasmic Ca^{2+} (Hong-Geller and Cerione, 2000). Therefore, regulation of Rac-1 activity will not be observed if PMA/ionomycin are used for stimulation. It also remains unclear how Notch activation specifically regulates IFNγ secretion without influencing the release of IL-2.

Final remarks

The use of L cells as artificial APCs is very convenient because they deliver "signal 1" and "signal 2" via MHC/peptide complexes and B7.1, respectively. However, for a more physiological examination of the role of Notch ligands during a MLR, professional APCs such as DCs overexpressing the ligands will be very useful. This can be studied in future using the transgenic mice with inducible expression of Jagged1 or Delta1 in DCs (chapter VI). Furthermore, these mice will allow to conduct *in vivo* studies of the role of Jagged1 or Delta1 during the initiation of an immune response.

Overall the work reported in this thesis has attempted to gain further insight into the role of Notch signalling during the induction of an immune response. I have shown that Notch ligands and receptors are expressed in DCs and T lymphocytes. However, I would like to emphasise that these demonstrations only represent gene expression studies and that further regulation occurring post-transcriptionally cannot be excluded.

Delta1 and Jagged1 expressed on APCs are capable to activate Notch in T cells. Jagged1-induced Notch signalling downregulates the secretion of IFNγ by T lymphocytes activated in a MLR, which may be one of the mechanisms responsible for the tolerance induced by Jagged1 transfected APCs *in vivo* (Hoyne *et al.*, 2000). I believe future experiments using transgenic mice with inducible overexpression of Jagged1 or Delta1 in DCs will provide a clearer understanding of the questions I have tried to address.

VIII References

Acuto, O., and Cantrell, D. (2000). T cell activation and the cytoskeleton. *Ann Rev Immunol* **18,** 165.

Adachi, O., Kawai, T., Takeda, K., Matsumoto, M. *et al.* (1998). Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9,** 143.

Adams, D. H., Hubscher, S. G., Fisher, N. C., Williams, A. *et al.* (1996). Expression of E-selectin and E-selectin ligands in human liver inflammation. *Hepatology* **24,** 533.

Adams, D. H., and Lloyd, A. R. (1997). Chemokines: leucocyte recruitment and activation cytokines. *Lancet* **349,** 490.

Agarwal, S., and Rao, A. (1998). Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* **9,** 765.

Agger, R., Crowley, M. T., and Witmer-Pack, M. D. (1990). The surface of dendritic cells in the mouse as studied with monoclonal antibodies. *Int Rev Immunol* **6,** 89.

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I. L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404,** 193.

Akbari, O., DeKruyff, R. H., and Umetsu, D. T. (2001). Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol* **2,** 725.

Akira, S., Takeda, K., and Kaisho, T. (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* **2,** 675.

Alberola-Ila, J., Takaki, S., Kerner, J. D., and Perlmutter, R. M. (1997). Differential signaling by lymphocyte antigen receptors. *Ann Rev Immunol* **15,** 125.

Albert, M. L., Jegathesan, M., and Darnell, R. B. (2001). Dendritic cell maturation is required for the cross-tolerization of CD8+ T cells. *Nat Immunol* **2,** 1010.

Albert, M. L., Pearce, S. F., Francisco, L. M., Sauter, B. *et al.* (1998a). Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J Exp Med* **188,** 1359.

Albert, M. L., Sauter, B., and Bhardwaj, N. (1998b). Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392,** 86.

Allen, J. M., Forbush, K. A., and Perlmutter, R. M. (1992). Functional dissection of the lck proximal promoter. *Mol Cell Biol* **12,** 2758.

Allen, R. T., Hunter, W. J., and Agrawal, D. K. (1997). Morphological and biochemical characterization and analysis of apoptosis. *J Pharmacol Toxicol Methods* **37,** 215.

Allman, D., Karnell, F. G., Punt, J. A., Bakkour, S. *et al.* (2001). Separation of Notch1 promoted lineage commitment and expansion/transformation in developing T cells. *J Exp Med* **194,** 99.

Altmann, D., Hogg, N., Trowsdale, J., and Wilkinson, D. (1989). Cotransfection of ICAM-1 and HLA-DR reconstitutes human antigen-presenting cell function in mouse L cells. *Nature* **338,** 512.

Anderson, C. C., and Matzinger, P. (2001). Immunity or tolerance: opposite outcomes of microchimerism from skin grafts. *Nat Med* **7,** 80.

Androlewicz, M. J. (2001). Peptide generation in the major histocompatibility complex class I antigen processing and presentation pathway. *Curr Opin Hematol* **8,** 12.

Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M. E. (1995). Notch signaling. *Science* **268,** 225.

Aste-Amezaga, M., Ma, X., Sartori, A., and Trinchieri, G. (1998). Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. *J Immunol* **160,** 5936.

Aster, J. C., Xu, L., Karnell, F. G., Patriub, V. *et al.* (2000). Essential roles for ankyrin repeat and transactivation domains in induction of T-cell leukemia by notch1. *Mol Cell Biol* **20,** 7505.

Austin, C. P., Feldman, D. E., Ida, J. A. J., and Cepko, C. L. (1995). Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. *Development* **121,** 3637.

Bach, E. A., Aguet, M., and Schreiber, R. D. (1997). The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Ann Rev Immunol* **15,** 563.

Bae, S., Bessho, Y., Hojo, M., and Kageyama, R. (2000). The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. *Development* **127,** 2933.

Baetz, K., Isaaz, S., and Griffiths, G. M. (1995). Loss of cytotoxic T lymphocyte function in Chediak-Higashi syndrome arises from a secretory defect that prevents lytic granule exocytosis. *J Immunol* **154,** 6122.

Bain, G., Robanus Maandag, E. C., te Riele, H. P., Feeney, A. J. *et al.* (1997). Both E12 and E47 allow commitment to the B cell lineage. *Immunity* **6,** 145.

Baldwin, A. S. (1996). The NF-kappa B and I kappa B proteins: new discoveries and insights. *Ann Rev Immunol* **14,** 649.

Banchereau, J., Briere, F., Caux, C., Davoust, J. *et al.* (2000). Immunobiology of dendritic cells. *Ann Rev Immunol* **18,** 767.

Barclay, A. N., and Mayrhofer, G. (1981). Bone marrow origin of Ia-positive cells in the medulla rat thymus. *J Exp Med* **153,** 1666.

Baroja, M. L., Lorre, K., Van Vaeck, F., and Ceuppens, J. L. (1989). The anti-T cell monoclonal antibody 9.3 (anti-CD28) provides a helper signal and bypasses the need for accessory cells in T cell activation with immobilized anti-CD3 and mitogens. *Cell Immunol* **120,** 205.

Baron, U., Freundlieb, S., Gossen, M., and Bujard, H. (1995). Co-regulation of two gene activities by tetracycline via a bidirectional promoter. *Nucl Acids Res* **23,** 3605.

Bash, J., Zong, W. X., Banga, S., Rivera, A. *et al.* (1999). Rel/NF-kappaB can trigger the Notch signaling pathway by inducing the expression of Jagged1, a ligand for Notch receptors. *EMBO J* **18,** 2803.

Bates, E. E., Fournier, N., Garcia, E., Valladeau, J. *et al.* (1999). APCs express DCIR, a novel C-type lectin surface receptor containing an immunoreceptor tyrosine-based inhibitory motif. *J Immunol* **163,** 1973.

Bell, D., Young, J. W., and Banchereau, J. (1999). Dendritic cells. *Adv Immunol* **72,** 255.

Bellavia, D., Campese, A. F., Alesse, E., Vacca, A. *et al.* (2000). Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice. *EMBO J* **19,** 3337.

Bellinghausen, I., Brand, U., Steinbrink, K., Enk, A. H. *et al.* (2001). Inhibition of human allergic T-cell responses by IL-10-treated dendritic cells: differences from hydrocortisone-treated dendritic cells. *J Allergy Clin Immunol* **108,** 242.

Bellone, M. (2000). Apoptosis, cross-presentation, and the fate of the antigen specific immune response. *Apoptosis* **5,** 307.

Bennett, S. R., Carbone, F. R., Karamalis, F., Flavell, R. A. *et al.* (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* **393,** 478.

Bessho, Y., Miyoshi, G., Sakata, R., and Kageyama, R. (2001). Hes7: a bHLH-type repressor gene regulated by Notch and expressed in the presomitic mesoderm. *Genes to Cells* **6,** 175.

Bettenhausen, B., Hrabe de Angelis, M., Simon, D., Guénet, J. L. *et al.* (1995). Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to *Drosophila* Delta. *Development* **121,** 2407.

Bieber, T., Rieger, A., Neuchrist, C., Prinz, J. C. *et al.* (1989). Induction of Fc epsilon R2/CD23 on human epidermal Langerhans cells by human recombinant interleukin 4 and gamma interferon. *J Exp Med* **170,** 309.

Bigas, A., Martin, D. I., and Milner, L. A. (1998). Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. *Mol Cell Biol* **18,** 2324.

Bird, J. J., Brown, D. R., Mullen, A. C., Moskowitz, N. H. *et al.* (1998). Helper T cell differentiation is controlled by the cell cycle. *Immunity* **9,** 229.

Blair, P. J., Riley, J. L., Levine, B. L., Lee, K. P. *et al.* (1998). CTLA-4 ligation delivers a unique signal to resting human CD4 T cells that inhibits interleukin-2 secretion but allows Bcl-X(L) induction. *J Immunol* **160,** 12.

Blaumueller, C. M., Qi, H., Zagouras, P., and Artavanis-Tsakonas, S. (1997). Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. *Cell* **90,** 281.

Blauvelt, A., Katz, S. I., and Udey, M. C. (1995). Human Langerhans cells express E-cadherin. *J Invest Dermatol* **104,** 293.

Boise, L. H., Minn, A. J., Noel, P. J., June, C. H. *et al.* (1995). CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity* **3,** 87.

Bowen, K. M., Andrus, L., and Lafferty, K. J. (1980). Successful allotransplantation of mouse pancreatic islets to nonimmunosuppressed recipients. *Diabetes* **29 Suppl 1,** 98.

Brocker, T., Gulbranson-Judge, A., Flynn, S., Riedinger, M. *et al.* (1999). CD4 T cell traffic control: in vivo evidence that ligation of OX40 on CD4 T cells by OX40-ligand expressed on dendritic cells leads to the accumulation of CD4 T cells in B follicles. *Eur J Immunol* **29,** 1610.

Brocker, T., Riedinger, M., and Karjalainen, K. (1997). Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo. *J Exp Med* **185,** 541.

Brossart, P., Zobywalski, A., Grunebach, F., Behnke, L. *et al.* (2000). Tumor necrosis factor alpha and CD40 ligand antagonize the inhibitory effects of interleukin 10 on T-cell stimulatory capacity of dendritic cells. *Cancer Res* **60,** 4485.

Brou, C., Logeat, F., Gupta, N., Bessia, C. *et al.* (2000). A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol Cell* **5,** 207.

Bruckner, K., Perez, L., Clausen, H., and Cohen, S. (2000). Glycosyltransferase activity of Fringe modulates Notch-Delta interactions [see comments]. *Nature* **406,** 411.

Brunner, C., Seiderer, J., Schlamp, A., Bidlingmaier, M. *et al.* (2000). Enhanced dendritic cell maturation by TNF-alpha or cytidine-phosphate-guanosine DNA drives T cell activation in vitro and therapeutic anti-tumor immune responses in vivo. *J Immunol* **165,** 6278.

Buckles, G. R., Rauskolb, C., Villano, J. L., and Katz, F. N. (2001). Four-jointed interacts with dachs, abelson and enabled and feeds back onto the Notch pathway to affect growth and segmentation in the *Drosophila* leg. *Development* **128,** 3533.

Buelens, C., Verhasselt, V., De Groote, D., Thielemans, K. *et al.* (1997). Human dendritic cell responses to lipopolysaccharide and CD40 ligation are differentially regulated by interleukin-10. *Eur J Immunol* **27,** 1848.

Buelens, C., Willems, F., Delvaux, A., Pierard, G. *et al.* (1995). Interleukin-10 differentially regulates B7-1 (CD80) and B7-2 (CD86) expression on human peripheral blood dendritic cells. *Eur J Immunol* **25,** 2668.

Bujdoso, R., Hopkins, J., Dutia, B. M., Young, P. *et al.* (1989). Characterization of sheep afferent lymph dendritic cells and their role in antigen carriage. *J Exp Med* **170,** 1285.

Bush, G., diSibio, G., Miyamoto, A., Denault, J. B. *et al.* (2001). Ligand-induced signaling in the absence of furin processing of Notch1. *Dev Biol* **229,** 494.

Callahan, J., Aster, J., Sklar, J., Kieff, E. *et al.* (2000). Intracellular forms of human NOTCH1 interact at distinctly different levels with RBP-jkappa in human B and T cells. *Leukemia* **14,** 84.

Calne, R. Y., Sells, R. A., Pena, J. R., Davis, D. R. *et al.* (1969). Induction of immunological tolerance by porcine liver allografts. *Nature* **223,** 472.

Capobianco, A. J., Zagouras, P., Blaumueller, C. M., Artavanis-Tsakonas, S. *et al.* (1997). Neoplastic transformation by truncated alleles of human NOTCH1/TAN1 and NOTCH2. *Mol Cell Biol* **17,** 6265.

Carlesso, N., Aster, J. C., Sklar, J., and Scadden, D. T. (1999). Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics. *Blood* **93,** 838.

Carroll, M. C. (1998). The role of complement and complement receptors in induction and regulation of immunity. *Ann Rev Immunol* **16,** 545.

Carter, L. L., and Murphy, K. M. (1999). Lineage-specific requirement for signal transducer and activator of transcription (Stat)4 in interferon gamma production from CD4(+) versus CD8(+) T cells. *J Exp Med* **189,** 1355.

Cascieri, M. A., and Springer, M. S. (2000). The chemokine/chemokine-receptor family: potential and progress for therapeutic intervention. *Curr Opin Immunol* **4,** 420.

Castella, P., Wagner, J. A., and Caudy, M. (1999). Regulation of hippocampal neuronal differentiation by the basic helix-loop-helix transcription factors HES-1 and MASH-1. *J Neurosci Res* **56,** 229.

Caux, C., Vanbervliet, B., Massacrier, C., Azuma, M. *et al.* (1994). B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *J Exp Med* **180,** 1841.

Caux, C., Vanbervliet, B., Massacrier, C., Dezutter-Dambuyant, C. *et al.* (1996). $CD34^+$ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. *J Exp Med* **184,** 695.

Cella, M., Salio, M., Sakakibara, Y., Langen, H. *et al.* (1999). Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J Exp Med* **189,** 821.

Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P. *et al.* (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med* **184,** 747.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. *et al.* (1994). Green fluorescent protein as a marker for gene expression. *Science* **263,** 802.

Chambers, C. A. (2001). The expanding world of co-stimulation: the two-signal model revisited. *Ann Rev Immunol* **22,** 217.

Chambers, C. A., Krummel, M. F., Boitel, B., Hurwitz, A. *et al.* (1996). The role of CTLA-4 in the regulation and initiation of T-cell responses. *Immunol Rev* **153,** 27.

Chambers, C. A., Kuhns, M. S., Egen, J. G., and Allison, J. P. (2001). CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Ann Rev Immunol* **19,** 565.

Chan, A. C., Iwashima, M., Turck, C. W., and Weiss, A. (1992). ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell* **71,** 649.

Chang, C. H., Furue, M., and Tamaki, K. (1995). B7-1 expression of Langerhans cells is up-regulated by proinflammatory cytokines, and is down-regulated by interferon-gamma or by interleukin-10. *Eur J Immunol* **25,** 394.

Chang, D., Valdez, P., Ho, T., and Robey, E. (2000). MHC recognition in thymic development: distinct, parallel pathways for survival and lineage commitment. *J Immunol* **165,** 6710.

Charlton, B., and Lafferty, K. J. (1995). The Th1/Th2 balance in autoimmunity. *Curr Opin Immunol* **7,** 793.

Cheng, P., Zlobin, A., Volgina, V., Gottipati, S. *et al.* (2001). Notch-1 regulates NF-kappaB activity in hemopoietic progenitor cells. *J Immunol* **167,** 4458.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. *et al.* (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the *Drosophila* neurogenic gene Delta [see comments]. *Nature* **375,** 761.

Chtanova, T., Kemp, R. A., Sutherland, A. P., Ronchese, F. *et al.* (2001). Gene microarrays reveal extensive differential gene expression in both cd4(+) and cd8(+) type 1 and type 2 t cells. *J Immunol* **167,** 3057.

Coccia, E. M., Passini, N., Battistini, A., Pini, C. *et al.* (1999). Interleukin-12 induces expression of interferon regulatory factor-1 via signal transducer and activator of transcription-4 in human T helper type 1 cells. *J Biol Chem* **274,** 6698.

Constant, S. L., and Bottomly, K. (1997). Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Ann Rev Immunol* **15,** 297.

Corinti, S., Albanesi, C., la Sala, A., Pastore, S. *et al.* (2001). Regulatory activity of autocrine IL-10 on dendritic cell functions. *J Immunol* **166,** 4312.

Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173,** 33.

Cox, D., Lee, D. J., Dale, B. M., Calafat, J. *et al.* (2000). A Rab11-containing rapidly recycling compartment in macrophages that promotes phagocytosis. *Proc Natl Acad Sci USA* **97,** 680.

Crameri, A., Whitehorn, E. A., Tate, E., and Stemmer, W. P. (1996). Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat Biotechnol* **14,** 315.

Craston, R., Koh, M., Mc Dermott, A., Ray, N. *et al.* (1997). Temporal dynamics of CD69 expression on lymphoid cells. *J Immunol Methods* **209,** 37.

Croft, M., Carter, L., Swain, S. L., and Dutton, R. W. (1994). Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J Exp Med* **180,** 1715.

Cruikshank, W. W., Berman, J. S., Theodore, A. C., Bernardo, J. *et al.* (1987). Lymphokine activation of T4+ T lymphocytes and monocytes. *J Immunol* **138,** 3817.

Cruikshank, W. W., Center, D. M., Nisar, N., Wu, M. *et al.* (1994). Molecular and functional analysis of a lymphocyte chemoattractant factor: association of biologic function with CD4 expression. *Proc Natl Acad Sci USA* **91,** 5109.

Damle, N. K., Klussman, K., Linsley, P. S., and Aruffo, A. (1992). Differential costimulatory effects of adhesion molecules B7, ICAM-1, LFA-3, and VCAM-1 on resting and antigen-primed CD4+ T lymphocytes. *J Immunol* **148,** 1985.

Danzer, S. G., Kirchner, H., and Rink, L. (1994). Cytokine interactions in human mixed lymphocyte culture. *Transplantation* **57,** 1638.

Davis, A. L., McKenzie, J. L., and Hart, D. N. (1988). HLA-DR-positive leucocyte subpopulations in human skin include dendritic cells, macrophages, and CD7-negative T cells. *Immunology* **65,** 573.

De Benedette, M. A., Shahinian, A., Mak, T. W., and Watts, T. H. (1997). Costimulation of CD28- T lymphocytes by 4-1BB ligand. *J Immunol* **158,** 551.

de Celis, J. F., and Bray, S. (1997). Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the *Drosophila* wing. *Development* **124,** 3241.

de Koster, H. S., Anderson, D. C., and Termijtelen, A. (1989). T cells sensitized to synthetic HLA-DR3 peptide give evidence of continuous presentation of denatured HLA-DR3 molecules by HLA-DP. *J Exp Med* **169,** 1191.

de Koster, H. S., van Rood, J. J., and Termijtelen, A. (1992). HLA-DR peptide-induced alloreactive T cell lines reveal an HLA-DR sequence that can be both "dominant" and "cryptic": evidence for allele-specific processing. *Eur J Immunol* **22,** 1531.

de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E. *et al.* (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124,** 1139.

de St Groth, B. F. (1998). The evolution of self-tolerance: a new cell arises to meet the challenge of self-reactivity. *Immunol Today* **19,** 448.

de St Groth, B. F., Smith, A. L., Koh, W. P., Girgis, L. *et al.* (1999). Carboxyfluorescein diacetate succinimidyl ester and the virgin lymphocyte: a marriage made in heaven. *Immunol Cell Biol* **77,** 530.

De Strooper, B., Annaert, W., Cupers, P., Saftig, P. *et al.* (1999). A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain [see comments]. *Nature* **398,** 518.

de Waal, L. P., Melief, C. J., and Melvold, R. W. (1981). Cytotoxic T lymphocytes generated across and I-Ab mutant difference are directed against a molecule bearing Ia antigens. *Eur J Immunol* **11,** 258.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G. *et al.* (1991). Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* **174,** 1209.

de Waal Malefyt, R., Yssel, H., and de Vries, J. E. (1993). Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells. Specific inhibition of IL-2 production and proliferation. *J Immunol* **150,** 4754.

Deftos, M. L., He, Y. W., Ojala, E. W., and Bevan, M. J. (1998). Correlating notch signaling with thymocyte maturation. *Immunity* **9,** 777.

Deftos, M. L., Huang, E., Ojala, E. W., Forbush, K. A. *et al.* (2000). Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes. *Immunity* **13,** 73.

Del Prete, G., De Carli, M., Almerigogna, F., Giudizi, M. G. *et al.* (1993). Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J Immunol* **150,** 353.

Dent, A. L., Shaffer, A. L., Yu, X., Allman, D. *et al.* (1997). Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science* **276,** 589.

Dieckmann, D., Plottner, H., Berchtold, S., Berger, T. *et al.* (2001). Ex vivo isolation and characterization of cd4(+)cd25(+) t cells with regulatory properties from human blood. *J Exp Med* **193,** 1303.

Diederich, R. J., Matsuno, K., Hing, H., and Artavanis-Tsakonas, S. (1994). Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. *Development* **120,** 473.

Dieu, M. C., Vanbervliet, B., Vicari, A., Bridon, J. M. *et al.* (1998). Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med* **188,** 373.

Doetschman, T., Maeda, N., and Smithies, O. (1988). Targeted mutation of the Hprt gene in mouse embryonic stem cells. *Proc Natl Acad Sci USA* **85,** 8583.

Doherty, D., Feger, G., Younger-Shepherd, S., Jan, L. Y. *et al.* (1996). Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in *Drosophila* wing formation. *Genes Dev* **10,** 421.

Dornseifer, P., Takke, C., and Campos-Ortega, J. A. (1997). Overexpression of a zebrafish homologue of the *Drosophila* neurogenic gene Delta perturbs differentiation of primary neurons and somite development. *Mech Dev* **63,** 159.

d'Ostiani, C. F., Del Sero, G., Bacci, A., Montagnoli, C. *et al.* (2000). Dendritic cells discriminate between yeasts and hyphae of the fungus Candida albicans. Implications for initiation of T helper cell immunity in vitro and in vivo. *J Exp Med* **191,** 1661.

Dubois, P. M., Pihlgren, M., Tomkowiak, M., Van Mechelen, M. *et al.* (1998). Tolerant CD8 T cells induced by multiple injections of peptide antigen show impaired TCR signaling and altered proliferative responses in vitro and in vivo. *J Immunol* **161,** 5260.

Dye, C. A., Lee, J. K., Atkinson, R. C., Brewster, R. *et al.* (1998). The *Drosophila* sanpodo gene controls sibling cell fate and encodes a tropomodulin homolog, an actin/tropomyosin-associated protein. *Development* **125,** 1845.

Eberhard, D., Jimenez, G., Heavey, B., and Busslinger, M. (2000). Transcriptional repression by Pax5 (BSAP) through interaction with corepressors of the Groucho family. *EMBO J* **19,** 2292.

Edmead, C. E., Patel, Y. I., Wilson, A., Boulougouris, G. *et al.* (1996). Induction of activator protein (AP)-1 and nuclear factor-kappaB by CD28 stimulation involves both phosphatidylinositol 3-kinase and acidic sphingomyelinase signals. *J Immunol* **157,** 3290.

Egner, W., Andreesen, R., and Hart, D. N. (1993). Allostimulatory cells in fresh human blood: heterogeneity in antigen-presenting cell populations. *Transplantation* **56,** 945.

Egner, W., and Hart, D. N. (1995). The phenotype of freshly isolated and cultured human bone marrow allostimulatory cells: possible heterogeneity in bone marrow dendritic cell populations. *Immunology* **85,** 611.

Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L. *et al.* (1991). TAN-1, the human homolog of the *Drosophila* notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66,** 649.

Elmets, C. A., Bergstresser, P. R., Tigelaar, R. E., Wood, P. J. *et al.* (1983). Analysis of the mechanism of unresponsiveness produced by haptens painted on skin exposed to low dose ultraviolet radiation. *J Exp Med* **158,** 781.

Enk, A. H., Angeloni, V. L., Udey, M. C., and Katz, S. I. (1993). Inhibition of Langerhans cell antigen-presenting function by IL-10. A role for IL-10 in induction of tolerance. *J Immunol* **151,** 2390.

Enk, A. H., Jonuleit, H., Saloga, J., and Knop, J. (1997). Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. *Int J Cancer* **73,** 309.

Enk, A. H., and Katz, S. I. (1992). Identification and induction of keratinocyte-derived IL-10. *J Immunol* **149,** 92.

Fagnoni, F. F., Takamizawa, M., Godfrey, W. R., Rivas, A. *et al.* (1995). Role of B70/B7-2 in CD4+ T-cell immune responses induced by dendritic cells. *Immunology* **85,** 467.

Fairchild, P. J., and Austyn, J. M. (1990). Thymic dendritic cells: phenotype and function. *Int Rev Immunol* **6,** 187.

Fairchild, P. J., and Waldmann, H. (2000). Dendritic cells and prospects for transplantation tolerance. *Curr Opin Immunol* **12,** 528.

Fanger, N. A., Wardwell, K., Shen, L., Tedder, T. F. *et al.* (1996). Type I (CD64) and type II (CD32) Fc gamma receptor-mediated phagocytosis by human blood dendritic cells. *J Immunol* **157,** 541.

Fedoseyeva, E. V., Tam, R. C., Popov, I. A., Orr, P. L. *et al.* (1996). Induction of T cell responses to a self-antigen following allotransplantation. *Transplantation* **61,** 679.

Felli, M. P., Maroder, M., Mitsiadis, T. A., Campese, A. F. *et al.* (1999). Expression pattern of notch1, 2 and 3 and Jagged1 and 2 in lymphoid and stromal thymus components: distinct ligand-receptor interactions in intrathymic T cell development. *Int Immunol* **11,** 1017.

Fishman, G. I., Kaplan, M. L., and Buttrick, P. M. (1994). Tetracycline-regulated cardiac gene expression in vivo. *J Clin Invest* **93,** 1864.

Flavell, R. A., Li, B., Dong, C., Lu, H. T. *et al.* (1999). Molecular basis of T-cell differentiation. *Cold Spring Harb Symp Quant Biol* **64,** 563.

Fleming, R. J. (1998). Structural conservation of Notch receptors and ligands. *Semin Cell Dev Biol* **9,** 599.

Fleming, R. J., Gu, Y., and Hukriede, N. A. (1997). Serrate-mediated activation of Notch is specifically blocked by the product of the gene fringe in the dorsal compartment of the *Drosophila* wing imaginal disc. *Development* **124,** 2973.

Flynn, S., Toellner, K. M., Raykundalia, C., Goodall, M. *et al.* (1998). CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Blr-1. *J Exp Med* **188,** 297.

Fortini, M. E., and Artavanis-Tsakonas, S. (1994). The suppressor of hairless protein participates in notch receptor signaling. *Cell* **79,** 273.

Fossum, S. (1988). Lymph-borne dendritic leucocytes do not recirculate, but enter the lymph node paracortex to become interdigitating cells. *Scand J Immunol* **27,** 97.

Foxman, E. F., Campbell, J. J., and Butcher, E. C. (1997). Multistep navigation and the combinatorial control of leukocyte chemotaxis. *J Cell Biol* **139,** 1349.

Franc, N. C., White, K., and Ezekowitz, R. A. (1999). Phagocytosis and development: back to the future. *Curr Opin Immunol* **11,** 47.

Freeman, G. J., Borriello, F., Hodes, R. J., Reiser, H. *et al.* (1993). Uncovering of functional alternative CTLA-4 counter-receptor in B7-deficient mice. *Science* **262,** 907.

Freeman, G. J., Boussiotis, V. A., Anumanthan, A., Bernstein, G. M. *et al.* (1995). B7-1 and B7-2 do not deliver identical costimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. *Immunity* **2,** 523.

Freemont, P. S. (1993). The RING finger. A novel protein sequence motif related to the zinc finger. *Ann N Y Acad Sci* **684,** 174.

Fries, K. M., Sempowski, G. D., Gaspari, A. A., Blieden, T. *et al.* (1995). CD40 expression by human fibroblasts. *Clin Immunol Immunopathol* **77,** 42.

Frise, E., Knoblich, J. A., Younger-Shepherd, S., Jan, L. Y. *et al.* (1996). The *Drosophila* Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage. *Proc Natl Acad Sci USA* **93,** 11925.

Furukawa, T., Itoh, M., Krueger, N. X., Streuli, M. *et al.* (1994). Specific interaction of the CD45 protein-tyrosine phosphatase with tyrosine-phosphorylated CD3 zeta chain. *Proc Natl Acad Sci USA* **91,** 10928.

Galvin, F., Freeman, G. J., Razi-Wolf, Z., Hall, W. *et al.* (1992). Murine B7 antigen provides a sufficient costimulatory signal for antigen-specific and MHC-restricted T cell activation. *J Immunol* **149,** 3802.

Garcia, S., DiSanto, J., and Stockinger, B. (1999). Following the development of a CD4 T cell response in vivo: from activation to memory formation. *Immunity* **11,** 163.

Gately, M. K., Renzetti, L. M., Magram, J., Stern, A. S. *et al.* (1998). The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Ann Rev Immunol* **16,** 495.

Geijtenbeek, T. B., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C. *et al.* (2000). Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses [see comments]. *Cell* **100,** 575.

Germain, R. N., and Stefanova, I. (1999). The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Ann Rev Immunol* **17,** 467.

Gett, A. V., and Hodgkin, P. D. (1998). Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. *Proc Natl Acad Sci USA* **95,** 9488.

Ghosh, S., May, M. J., and Kopp, E. B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Ann Rev Immunol* **16,** 225.

Gimmi, C. D., Freeman, G. J., Gribben, J. G., Gray, G. *et al.* (1993). Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. *Proc Natl Acad Sci USA* **90,** 6586.

Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA* **89,** 5547.

Granucci, F., Vizzardelli, C., Virzi, E., Rescigno, M. *et al.* (2001). Transcriptional reprogramming of dendritic cells by differentiation stimuli. *Eur J Immunol* **31,** 2539.

Grbavec, D., and Stifani, S. (1996). Molecular interaction between TLE1 and the carboxyl-terminal domain of HES-1 containing the WRPW motif. *Biochem Biophys Res Commun* **223,** 701.

Grewal, I. S., and Flavell, R. A. (1998). CD40 and CD154 in cell-mediated immunity. *Ann Rev Immunol* **16,** 111.

Grouard, G., Rissoan, M. C., Filgueira, L., Durand, I. *et al.* (1997). The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J Exp Med* **185,** 1101.

Groux, H., Bigler, M., de Vries, J. E., and Roncarolo, M. G. (1996). Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J Exp Med* **184,** 19.

Groux, H., Bigler, M., de Vries, J. E., and Roncarolo, M. G. (1998). Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. *J Immunol* **160,** 3188.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M. *et al.* (1997). A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389,** 737.

Guan, E., Wang, J., Laborda, J., Norcross, M. *et al.* (1996). T cell leukemia-associated human Notch/translocation-associated Notch homologue has I kappa B-like activity and physically interacts with nuclear factor-kappa B proteins in T cells. *J Exp Med* **183,** 2025.

Guerder, S., and Matzinger, P. (1992). A fail-safe mechanism for maintaining self-tolerance. *J Exp Med* **176,** 553.

Gunn, M. D., Tangemann, K., Tam, C., Cyster, J. G. *et al.* (1998). A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc Natl Acad Sci USA* **95,** 258.

Guo, M., Gong, S., Maric, S., Misulovin, Z. *et al.* (2000). A monoclonal antibody to the DEC-205 endocytosis receptor on human dendritic cells. *Hum Immunol* **61,** 729.

Hadjantonakis, A. K., Gertsenstein, M., Ikawa, M., Okabe, M. *et al.* (1998). Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech Dev* **76,** 79.

Han, W., Ye, Q., and Moore, M. A. (2000). A soluble form of human Delta-like-1 inhibits differentiation of hematopoietic progenitor cells. *Blood* **95,** 1616.

Harden, N., Loh, H. Y., Chia, W., and Lim, L. (1995). A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in *Drosophila*. *Development* **121,** 903.

Harding, F. A., McArthur, J. G., Gross, J. A., Raulet, D. H. *et al.* (1992). CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* **356,** 607.

Hargreaves, R., Logiou, V., and Lechler, R. (1995). The primary alloresponse of human CD4+ T cells is dependent on B7 (CD80), augmented by CD58, but relatively uninfluenced by CD54 expression. *Int Immunol* **7,** 1505.

Hart, D. N., and Fabre, J. W. (1981). Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain. *J Exp Med* **154,** 347.

Hart, D. N., Fuggle, S. V., Williams, K. A., Fabre, J. W. *et al.* (1981). Localization of HLA-ABC and DR antigens in human kidney. *Transplantation* **31,** 428.

Hartmann, G., Weiner, G. J., and Krieg, A. M. (1999). CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc Natl Acad Sci USA* **96,** 9305.

Hasbold, J., Gett, A. V., Rush, J. S., Deenick, E. *et al.* (1999). Quantitative analysis of lymphocyte differentiation and proliferation in vitro using carboxyfluorescein diacetate succinimidyl ester. *Immunol Cell Biol* **77,** 516.

Hasbold, J., Lyons, A. B., Kehry, M. R., and Hodgkin, P. D. (1998). Cell division number regulates IgG1 and IgE switching of B cells following stimulation by CD40 ligand and IL-4. *Eur J Immunol* **28,** 1040.

Hashimoto, S., Suzuki, T., Dong, H. Y., Nagai, S. *et al.* (1999). Serial analysis of gene expression in human monocyte-derived dendritic cells. *Blood* **94,** 845.

Hasserjian, R. P., Aster, J. C., Davi, F., Weinberg, D. S. *et al.* (1996). Modulated expression of notch1 during thymocyte development. *Blood* **88,** 970.

Hawrylowicz, C., Guida, L., Jarman, E., Hoyne, G. *et al.* (1995). Modulation of immune responses to allergens of house dust mite. *Biochem Soc Trans* **23,** 660.

Heim, R., and Tsien, R. Y. (1996). Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr Biol* **6,** 178.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C. *et al.* (1996). Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in *Drosophila*. *Development* **122,** 161.

Henrique, D., Hirsinger, E., Adam, J., Le Roux, I. *et al.* (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. *Curr Biol* **7,** 661.

Herndon, T. M., Shan, X. C., Tsokos, G. C., and Wange, R. L. (2001). ZAP-70 and SLP-76 regulate protein kinase C-theta and NF-kappa B activation in response to engagement of CD3 and CD28. *J Immunol* **166,** 5654.

Heufler, C., Koch, F., Stanzl, U., Topar, G. *et al.* (1996). Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. *Eur J Immunol* **26,** 659.

Hickling, J. K., Fenton, C. M., Howland, K., Marsh, S. G. *et al.* (1990). Peptides recognized by class I restricted T cells also bind to MHC class II molecules. *Int Immunol* **2,** 435.

Hicks, C., Johnston, S. H., di Sibio, G., Collazo, A. *et al.* (2000). Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. *Nat Cell Biol* **2,** 515.

Higuchi, R., Dollinger, G., Walsh, P. S., and Griffith, R. (1992). Simultaneous amplification and detection of specific DNA sequences. *Biotechnology* **10,** 413.

Ho, I. C., Hodge, M. R., Rooney, J. W., and Glimcher, L. H. (1996). The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. *Cell* **85,** 973.

Ho, I. C., Lo, D., and Glimcher, L. H. (1998). c-maf promotes T helper cell type 2 (Th2) and attenuates Th1 differentiation by both interleukin 4-dependent and -independent mechanisms. *J Exp Med* **188,** 1859.

Hogg, N., Takacs, L., Palmer, D. G., Selvendran, Y. *et al.* (1986). The p150,95 molecule is a marker of human mononuclear phagocytes: comparison with expression of class II molecules. *Eur J Immunol* **16,** 240.

Holland, P. M., Abramson, R. D., Watson, R., and Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci USA* **88,** 7276.

Hong-Geller, E., and Cerione, R. A. (2000). Cdc42 and Rac stimulate exocytosis of secretory granules by activating the IP(3)/calcium pathway in RBL-2H3 mast cells. *J Cell Biol* **148,** 481.

Hoyne, G. F., Callow, M. G., Kuo, M. C., and Thomas, W. R. (1993a). Characterization of T-cell responses to the house dust mite allergen Der p II in mice. Evidence for major and cryptic epitopes. *Immunology* **78,** 65.

Hoyne, G. F., Callow, M. G., Kuo, M. C., and Thomas, W. R. (1993b). Comparison of antigen presentation by lymph node cells from protein and peptide-primed mice. *Immunology* **78,** 58.

Hoyne, G. F., Dallman, M. J., and Lamb, J. R. (1999). Linked suppression in peripheral T cell tolerance to the house dust mite derived allergen Der p 1. *Int Arch Allergy Immunol* **118,** 122.

Hoyne, G. F., Jarnicki, A. G., Thomas, W. R., and Lamb, J. R. (1997). Characterization of the specificity and duration of T cell tolerance to intranasally administered peptides in mice: a role for intramolecular epitope suppression. *Int Immunol* **9,** 1165.

Hoyne, G. F., Le Roux, I., Corsin-Jimenez, M., Tan, K. *et al.* (2000). Serrate1-induced notch signalling regulates the decision between immunity and tolerance made by peripheral CD4(+) T cells. *Int Immunol* **12,** 177.

Hoyne, G. F., O'Hehir, R. E., Wraith, D. C., Thomas, W. R. *et al.* (1993c). Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. *J Exp Med* **178,** 1783.

Hsieh, J. J., Zhou, S., Chen, L., Young, D. B. *et al.* (1999). CIR, a corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex. *Proc Natl Acad Sci USA* **96,** 23.

Huang, F. P., Platt, N., Wykes, M., Major, J. R. *et al.* (2000). A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes [see comments]. *J Exp Med* **191,** 435.

Huang, H., Hu Li, J., Chen, H., Ben Sasson, S. Z. *et al.* (1997). IL-4 and IL-13 production in differentiated T helper type 2 cells is not IL-4 dependent. *J Immunol* **159,** 3731.

Huang, H., and Paul, W. E. (1998). Impaired interleukin 4 signaling in T helper type 1 cells. *J Exp Med* **187,** 1305.

Hurme, M., Henttinen, T., Karppelin, M., Varkila, K. *et al.* (1994). Effect of interleukin-10 on NF-kB and AP-1 activities in interleukin-2 dependent CD8 T lymphoblasts. *Immunol Lett* **42,** 129.

Hussain, L. A., and Lehner, T. (1995). Comparative investigation of Langerhans' cells and potential receptors for HIV in oral, genitourinary and rectal epithelia. *Immunology* **85,** 475.

Inaba, K., Inaba, M., Romani, N., Aya, H. *et al.* (1992a). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* **176,** 1693.

Inaba, K., Metlay, J. P., Crowley, M. T., and Steinman, R. M. (1990). Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ. *J Exp Med* **172,** 631.

Inaba, K., Pack, M., Inaba, M., Sakuta, H. *et al.* (1997). High levels of a major histocompatibility complex II-self peptide complex on dendritic cells from the T cell areas of lymph nodes. *J Exp Med* **186,** 665.

Inaba, K., Steinman, R. M., Pack, M. W., Aya, H. *et al.* (1992b). Identification of proliferating dendritic cell precursors in mouse blood. *J Exp Med* **175,** 1157.

Inaba, K., Turley, S., Yamaide, F., Iyoda, T. *et al.* (1998). Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J Exp Med* **188,** 2163.

Ishizaka, S., Saito, S., Yoshikawa, M., Kimoto, M. *et al.* (1996). IL-10 production in mouse hepatocytes augmented by TGF-beta. *Cytokine* **8,** 837.

Ito, T., Inaba, M., Inaba, K., Toki, J. *et al.* (1999). A CD1a+/CD11c+ subset of human blood dendritic cells is a direct precursor of Langerhans cells. *J Immunol* **163,** 1409.

Itoh, M., Takahashi, T., Sakaguchi, N., Kuniyasu, Y. *et al.* (1999). Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* **162,** 5317.

Iwasaki, A., and Kelsall, B. L. (1999). Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *J Exp Med* **190,** 229.

Izon, D. J., Punt, J. A., Xu, L., Karnell, F. G. *et al.* (2001). Notch1 regulates maturation of CD4+ and CD8+ thymocytes by modulating TCR signal strength. *Immunity* **14,** 253.

Jackson, I. J., and Abbott, C. M. (2000). "Mouse Genetics and Transgenics: A practical approach" 1 ed., Oxford University Press Inc, New York.

Jain, J., Loh, C., and Rao, A. (1995). Transcriptional regulation of the IL-2 gene. *Curr Opin Immunol* **7,** 333.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H. *et al.* (1995). Signalling downstream of activated mammalian Notch [see comments]. *Nature* **377,** 355.

Jarriault, S., Le Bail, O., Hirsinger, E., Pourquié, O. *et al.* (1998). Delta-1 activation of notch-1 signaling results in HES-1 transactivation. *Mol Cell Biol* **18,** 7423.

Jehn, B. M., Bielke, W., Pear, W. S., and Osborne, B. A. (1999). Protective effects of notch-1 on TCR-induced apoptosis. *J Immunol* **162,** 635.

Jenkins, M. K., and Schwartz, R. H. (1987). Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J Exp Med* **165,** 302.

Jiang, W., Swiggard, W. J., Heufler, C., Peng, M. *et al.* (1995). The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature* **375,** 151.

Jones, P., May, G., Healy, L., Brown, J. *et al.* (1998). Stromal expression of Jagged 1 promotes colony formation by fetal hematopoietic progenitor cells. *Blood* **92,** 1505.

Jonuleit, H., Schmitt, E., Schuler, G., Knop, J. *et al.* (2000). Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* **192,** 1213.

Jonuleit, H., Schmitt, E., Stassen, M., Tuettenberg, A. *et al.* (2001). Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* **193,** 1285.

Josien, R., Heslan, M., Brouard, S., Soulillou, J. P. *et al.* (1998). Critical requirement for graft passenger leukocytes in allograft tolerance induced by donor blood transfusion. *Blood* **92,** 4539.

Josien, R., Wong, B. R., Li, H. L., Steinman, R. M. *et al.* (1999). TRANCE, a TNF family member, is differentially expressed on T cell subsets and induces cytokine production in dendritic cells. *J Immunol* **162,** 2562.

Ju, B. G., Jeong, S., Bae, E., Hyun, S. *et al.* (2000). Fringe forms a complex with Notch. *Nature* **405,** 191.

Julius, M. H., Simpson, E., and Herzenberg, L. A. (1973). A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur J Immunol* **3,** 645.

June, C. H., Bluestone, J. A., Nadler, L. M., and Thompson, C. B. (1994). The B7 and CD28 receptor families. *Immunol Today* **15,** 321.

Kadowaki, N., Antonenko, S., Ho, S., Rissoan, M. C. *et al.* (2001). Distinct cytokine profiles of neonatal natural killer T cells after expansion with subsets of dendritic cells. *J Exp Med* **193,** 1221.

Kahlert, H., Grage-Griebenow, E., Stuwe, H. T., Cromwell, O. *et al.* (2000). T cell reactivity with allergoids: influence of the type of APC. *J Immunol* **165,** 1807.

Kalinski, P., Hilkens, C. M., Snijders, A., Snijdewint, F. G. *et al.* (1997). IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. *J Immunol* **159,** 28.

Kalinski, P., Hilkens, C. M., Wierenga, E. A., and Kapsenberg, M. L. (1999). T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today* **20,** 561.

Kamada, N., Davies, H. S., and Roser, B. (1981). Reversal of transplantation immunity by liver grafting. *Nature* **292,** 840.

Kane, L. P., Andres, P. G., Howland, K. C., Abbas, A. K. *et al.* (2001). Akt provides the CD28 costimulatory signal for up-regulation of IL-2 and IFN-gamma but not TH2 cytokines. *Nat Immunol* **2,** 37.

Kaneta, M., Osawa, M., Sudo, K., Nakauchi, H. *et al.* (2000). A role for pref-1 and HES-1 in thymocyte development. *J Immunol* **164,** 256.

Kang, K., Hammerberg, C., Meunier, L., and Cooper, K. D. (1994). CD11b+ macrophages that infiltrate human epidermis after in vivo ultraviolet exposure potently produce IL-10 and represent the major secretory source of epidermal IL-10 protein. *J Immunol* **153,** 5256.

Kao, H. Y., Ordentlich, P., Koyano-Nakagawa, N., Tang, Z. *et al.* (1998). A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. *Genes Dev* **12,** 2269.

Kaplan, G., Walsh, G., Guido, L. S., Meyn, P. *et al.* (1992). Novel responses of human skin to intradermal recombinant granulocyte/macrophage-colony-stimulating factor: Langerhans cell recruitment, keratinocyte growth, and enhanced wound healing. *J Exp Med* **175,** 1717.

Kaplan, M. H., Wurster, A. L., and Grusby, M. J. (1998). A signal transducer and activator of transcription (Stat)4-independent pathway for the development of T helper type 1 cells. *J Exp Med* **188,** 1191.

Karanu, F. N., Murdoch, B., Miyabayashi, T., Ohno, M. *et al.* (2001). Human homologues of Delta-1 and Delta-4 function as mitogenic regulators of primitive human hematopoietic cells. *Blood* **97,** 1960.

Kato, M., Yamanouchi, K., Ikawa, M., Okabe, M. *et al.* (1999). Efficient selection of transgenic mouse embryos using EGFP as a marker gene. *Mol Reprod Dev* **54,** 43.

Katz, S. I., Tamaki, K., and Sachs, D. H. (1979). Epidermal Langerhans cells are derived from cells originating in bone marrow. *Nature* **282,** 324.

Kilby, N. J., Snaith, M. R., and Murray, J. A. (1993). Site-specific recombinases: tools for genome engineering. *Trends Genet* **9,** 413.

Kim, H. K., and Siu, G. (1998). The notch pathway intermediate HES-1 silences CD4 gene expression. *Mol Cell Biol* **18,** 7166.

Kim, Y. J., Kim, S. H., Mantel, P., and Kwon, B. S. (1998). Human 4-1BB regulates CD28 co-stimulation to promote Th1 cell responses. *Eur J Immunol* **28,** 881.

Kishi, N., Tang, Z., Maeda, Y., Hirai, A. *et al.* (2001). Murine homologs of deltex define a novel gene family involved in vertebrate Notch signaling and neurogenesis. *Int J Dev Neurosci* **19,** 21.

Kisielow, P., and von Boehmer, H. (1995). Development and selection of T cells: facts and puzzles. *Adv Immunol* **58,** 87.

Kistner, A., Gossen, M., Zimmermann, F., Jerecic, J. *et al.* (1996). Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc Natl Acad Sci USA* **93,** 10933.

Kitamura, M. (1996). Creation of a reversible on/off system for site-specific in vivo control of exogenous gene activity in the renal glomerulus. *Proc Natl Acad Sci USA* **93,** 7387.

Klasen, S., Pages, F., Peyron, J. F., Cantrell, D. A. *et al.* (1998). Two distinct regions of the CD28 intracytoplasmic domain are involved in the tyrosine phosphorylation of Vav and GTPase activating protein-associated p62 protein. *Int Immunol* **10,** 481.

Koch, F., Stanzl, U., Jennewein, P., Janke, K. *et al.* (1996). High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J Exp Med* **184,** 741.

Kondo, M., Weissman, I. L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91,** 661.

Koszik, F., Strunk, D., Simonitsch, I., Picker, L. J. *et al.* (1994). Expression of monoclonal antibody HECA-452-defined E-selectin ligands on Langerhans cells in normal and diseased skin. *J Invest Dermatol* **102,** 773.

Kouskoff, V., Fehling, H. J., Lemeur, M., Benoist, C. *et al.* (1993). A vector driving the expression of foreign cDNAs in the MHC class II-positive cells of transgenic mice. *J Immunol Methods* **166,** 287.

Krebs, D. L., and Hilton, D. J. (2001). Socs proteins: negative regulators of cytokine signaling. *Stem Cells* **19,** 378.

Kronenberg, S., Lai, Z., and Esser, C. (2000). Generation of alphabeta T-cell receptor+ CD4- CD8+ cells in major histocompatibility complex class I-deficient mice upon activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Immunology* **100,** 185.

Kronin, V., Winkel, K., Süss, G., Kelso, A. *et al.* (1996). A subclass of dendritic cells regulates the response of naive CD8 T cells by limiting their IL-2 production. *J Immunol* **157,** 3819.

Kronin, V., Wu, L., Gong, S., Nussenzweig, M. C. *et al.* (2000). DEC-205 as a marker of dendritic cells with regulatory effects on CD8 T cell responses. *Int Immunol* **12,** 731.

Krummel, M. F., and Allison, J. P. (1995). CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* **182,** 459.

Kuchroo, V. K., Das, M. P., Brown, J. A., Ranger, A. M. *et al.* (1995). B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* **80,** 707.

Kunisch, M., Haenlin, M., and Campos-Ortega, J. A. (1994). Lateral inhibition mediated by the *Drosophila* neurogenic gene delta is enhanced by proneural proteins. *Proc Natl Acad Sci USA* **91,** 10139.

Kuo, C. T., and Leiden, J. M. (1999). Transcriptional regulation of T lymphocyte development and function. *Ann Rev Immunol* **17,** 149.

Kuroda, K., Tani, S., Tamura, K., Minoguchi, S. *et al.* (1999). Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. *J Biol Chem* **274,** 7238.

Kurooka, H., and Honjo, T. (2000). Functional interaction between the mouse notch1 intracellular region and histone acetyltransferases PCAF and GCN5. *J Biol Chem* **275,** 17211.

Kurooka, H., Kuroda, K., and Honjo, T. (1998). Roles of the ankyrin repeats and C-terminal region of the mouse notch1 intracellular region. *Nucleic Acids Res* **26,** 5448.

Kurts, C., Kosaka, H., Carbone, F. R., Miller, J. F. *et al.* (1997). Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. *J Exp Med* **186,** 239.

Lam, L. T., Ronchini, C., Norton, J., Capobianco, A. J. *et al.* (2000). Suppression of erythroid but not megakaryocytic differentiation of human K562 erythroleukemic cells by notch-1. *J Biol Chem* **275,** 19676.

Lamb, J. R., Skidmore, B. J., Green, N., Chiller, J. M. *et al.* (1983). Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. *J Exp Med* **157,** 1434.

Lane, P. (2000). Role of OX40 signals in coordinating CD4 T cell selection, migration, and cytokine differentiation in T helper (Th)1 and Th2 cells. *J Exp Med* **191,** 201.

Langenkamp, A., Messi, M., Lanzavecchia, A., and Sallusto, F. (2000). Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat Immunol* **1,** 311.

Lanzavecchia, A., and Sallusto, F. (2001a). The instructive role of dendritic cells on T cell responses: lineages, plasticity and kinetics. *Curr Opin Immunol* **13,** 291.

Lanzavecchia, A., and Sallusto, F. (2001b). Regulation of T cell immunity by dendritic cells. *Cell* **106,** 263.

Lardelli, M., Dahlstrand, J., and Lendahl, U. (1994). The novel Notch homologue mouse Notch 3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. *Mech Dev* **46,** 123.

Larsson, M., Fonteneau, J. F., and Bhardwaj, N. (2001). Dendritic cells resurrect antigens from dead cells. *Trends Immunol* **22,** 141.

Lechler, R. I., Norcross, M. A., and Germain, R. N. (1985). Qualitative and quantitative studies of antigen-presenting cell function by using I-A-expressing L cells. *J Immunol* **135,** 2914.

Ledbetter, J. A., and Linsley, P. S. (1992). CD28 receptor crosslinking induces tyrosine phosphorylation of PLC gamma 1. *Adv Exp Med Biol* **323,** 23.

Lee, R. S., Rusche, J. R., Maloney, M. E., Sachs, D. H. *et al.* (2001). CTLA4Ig-induced linked regulation of allogeneic T cell responses. *J Immunol* **166,** 1572.

Lee, W. C., Zhong, C., Qian, S., Wan, Y. *et al.* (1998). Phenotype, function, and in vivo migration and survival of allogeneic dendritic cell progenitors genetically engineered to express TGF-beta. *Transplantation* **66,** 1810.

Leiva, L. E., Regueira, O., and Sorensen, R. U. (1997). Peripheral blood mononuclear cell sonicates as an alternative to irradiated allogeneic cells to stimulate a mixed lymphocyte reaction and to enumerate CD69+ alloreactive T cells. *Hum Immunol* **56,** 49.

Lenschow, D. J., Zeng, Y., Thistlethwaite, J. R., Montag, A. *et al.* (1992). Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science* **257,** 789.

Li, J., Peet, G. W., Balzarano, D., Li, X. *et al.* (2001). Novel NEMO/IkappaB kinase and NF-kappa B target genes at the pre-B to immature B cell transition. *J Biol Chem* **276,** 18579.

Li, L., Milner, L. A., Deng, Y., Iwata, M. *et al.* (1998). The human homolog of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. *Immunity* **8,** 43.

Lindsten, T., June, C. H., Ledbetter, J. A., Stella, G. *et al.* (1989). Regulation of lymphokine messenger RNA stability surface/mediated T cell activation pathway. *Science* **244,** 339.

Linsley, P. S., Bradshaw, J., Greene, J., Peach, R. *et al.* (1996). Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. *Immunity* **4,** 535.

Liu, Y. J. (2001). Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* **106,** 259.

Liu, Z. G., Smith, S. W., McLaughlin, K. A., Schwartz, L. M. *et al.* (1994). Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. *Nature* **367,** 281.

Logeat, F., Bessia, C., Brou, C., LeBail, O. *et al.* (1998). The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proc Natl Acad Sci USA* **95,** 8108.

Lohoff, M., Ferrick, D., Mittrucker, H. W., Duncan, G. S. *et al.* (1997). Interferon regulatory factor-1 is required for a T helper 1 immune response in vivo. *Immunity* **6,** 681.

Lombardi, G., Sidhu, S., Batchelor, R., and Lechler, R. (1994). Anergic T cells as suppressor cells in vitro. *Science* **264,** 1587.

Losman, J. A., Chen, X. P., Hilton, D., and Rothman, P. (1999). Cutting edge: SOCS-1 is a potent inhibitor of IL-4 signal transduction. *J Immunol* **162,** 3770.

Lowell, S., and Watt, F. M. (2001). Delta regulates keratinocyte spreading and motility independently of differentiation. *Mech Dev* **107,** 133.

Lu, L., Li, W., Fu, F., Chambers, F. G. *et al.* (1997). Blockade of the CD40-CD40 ligand pathway potentiates the capacity of donor-derived dendritic cell progenitors to induce long-term cardiac allograft survival. *Transplantation* **64,** 1808.

Lu, L., Liang, X., Li, W., Chen, Z. *et al.* (2001). A novel subset of dendritic cells propagated from the liver promotes differentiation of T regulatory cells and enhances allograft survival. *Transplant Proc* **33,** 229.

Lu, L., Woo, J., Rao, A. S., Li, Y. *et al.* (1994). Propagation of dendritic cell progenitors from normal mouse liver using granulocyte/macrophage colony-stimulating factor and their maturational development in the presence of type-1 collagen. *J Exp Med* **179,** 1823.

Ludewig, B., Graf, D., Gelderblom, H. R., Becker, Y. *et al.* (1995). Spontaneous apoptosis of dendritic cells is efficiently inhibited by TRAP (CD40-ligand) and TNF-alpha, but strongly enhanced by interleukin-10. *Eur J Immunol* **25,** 1943.

Luo, B., Aster, J. C., Hasserjian, R. P., Kuo, F. *et al.* (1997). Isolation and functional analysis of a cDNA for human Jagged2, a gene encoding a ligand for the Notch1 receptor. *Mol Cell Biol* **17,** 6057.

Lutz, M. B., Kukutsch, N., Ogilvie, A. L., Rossner, S. *et al.* (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* **223,** 77.

Lyons, A. B., and Parish, C. R. (1994). Determination of lymphocyte division by flow cytometry. *J Immunol Methods* **171,** 131.

Macatonia, S. E., Hosken, N. A., Litton, M., Vieira, P. *et al.* (1995). Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol* **154,** 5071.

MacDonald, A. S., Straw, A. D., Dalton, N. M., and Pearce, E. J. (2002). Th2 Response Induction by Dendritic Cells: A Role for CD40. *J Immunol* **168,** 537.

Mader, S., and White, J. H. (1993). A steroid-inducible promoter for the controlled overexpression of cloned genes in eukaryotic cells. *Proc Natl Acad Sci USA* **90,** 5603.

Mahnke, K., Becher, E., Ricciardi-Castagnoli, P., Luger, T. A. *et al.* (1997). CD14 is expressed by subsets of murine dendritic cells and upregulated by lipopolysaccharide. *Adv Exp Med Biol* **417,** 145.

Maldonado-López, R., De Smedt, T., Michel, P., Godfroid, J. *et al.* (1999). CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J Exp Med* **189,** 587.

Maraskovsky, E., Brasel, K., Teepe, M., Roux, E. R. *et al.* (1996). Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J Exp Med* **184,** 1953.

Martell, M., Gomez, J., Esteban, J.I., Sauleda, S., et al. (1999). High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. *J Clin Microbiol* **37**, 327.

Matsuno, K., Diederich, R. J., Go, M. J., Blaumueller, C. M. *et al.* (1995). Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development* **121,** 2633.

Matsuno, K., Eastman, D., Mitsiades, T., Quinn, A. M. *et al.* (1998). Human deltex is a conserved regulator of Notch signalling. *Nature Genetics* **19,** 74.

Matsuno, K., Ezaki, T., Kudo, S., and Uehara, Y. (1996). A life stage of particle-laden rat dendritic cells in vivo: their terminal division, active phagocytosis, and translocation from the liver to the draining lymph. *J Exp Med* **183,** 1865.

Matsuno, K., Go, M. J., Sun, X., Eastman, D. S. *et al.* (1997). Suppressor of Hairless-independent events in Notch signaling imply novel pathway elements. *Development* **124,** 4265.

Matzinger, P. (1994). Tolerance, danger, and the extended family. *Ann Rev Immunol* **12,** 991.

Maurer, D., Fiebiger, S., Ebner, C., Reininger, B. *et al.* (1996). Peripheral blood dendritic cells express Fc epsilon RI as a complex composed of Fc epsilon RI alpha- and Fc epsilon RI gamma-chains and can use this receptor for IgE-mediated allergen presentation. *J Immunol* **157,** 607.

May, M. J., and Ghosh, S. (1998). Signal transduction through NF-kappa B. *Immunol Today* **19,** 80.

McKenzie, G. J., Emson, C. L., Bell, S. E., Anderson, S. *et al.* (1998). Impaired development of Th2 cells in IL-13-deficient mice. *Immunity* **9,** 423.

McLellan, A. D., and Kampgen, E. (2000). Functions of myeloid and lymphoid dendritic cells. *Immunol Lett* **72,** 101.

McLellan, A. D., Starling, G. C., Williams, L. A., Hock, B. D. *et al.* (1995). Activation of human peripheral blood dendritic cells induces the CD86 co-stimulatory molecule [published erratum appears in Eur J Immunol 1995 Dec;25(12):3525]. *Eur J Immunol* **25,** 2064.

McWilliam, A. S., Nelson, D., Thomas, J. A., and Holt, P. G. (1994). Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. *J Exp Med* **179,** 1331.

Menges, M., Rößner, S., Voigtländer, C., Schindler, H. *et al.* (2002). Repetitive Injections of Dendritic Cells Matured with Tumor Necrosis Factor α Induce Antigen-specific Protection of Mice from Autoimmunity. *Nat Immunol* **195,** 15.

Merad, M., Fong, L., Bogenberger, J., and Engleman, E. G. (2000). Differentiation of myeloid dendritic cells into CD8alpha-positive dendritic cells in vivo. *Blood* **96,** 1865.

Metlay, J. P., Witmer-Pack, M. D., Agger, R., Crowley, M. T. *et al.* (1990). The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J Exp Med* **171,** 1753.

Migliaccio, G., Migliaccio, A. R., Kreider, B. L., Rovera, G. *et al.* (1989). Selection of lineage-restricted cell lines immortalized at different stages of hematopoietic differentiation from the murine cell line 32D. *J Cell Biol* **109,** 833.

Miller, A., Lider, O., and Weiner, H. L. (1991). Antigen-driven bystander suppression after oral administration of antigens. *J Exp Med* **174,** 791.

Milner, L. A., and Bigas, A. (1999). Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation. *Blood* **93,** 2431.

Milner, L. A., Bigas, A., Kopan, R., Brashem-Stein, C. *et al.* (1996). Inhibition of granulocytic differentiation by mNotch1. *Proc Natl Acad Sci USA* **93,** 13014.

Milner, L. A., Kopan, R., Martin, D. I., and Bernstein, I. D. (1994). A human homologue of the *Drosophila* developmental gene, Notch, is expressed in CD34+ hematopoietic precursors. *Blood* **83,** 2057.

Min, W. P., Gorczynski, R., Huang, X. Y., Kushida, M. *et al.* (2000). Dendritic cells genetically engineered to express Fas ligand induce donor-specific hyporesponsiveness and prolong allograft survival. *J Immunol* **164,** 161.

Minami, Y., and Taniguchi, T. (1995). IL-2 signaling: recruitment and activation of multiple protein tyrosine kinases by the components of the IL-2 receptor. *Current Opinion in Cell Biology* **7,** 156.

Mizutani, K., Matsubayashi, T., Iwase, S., Doi, T. S. *et al.* (2000). Murine Delta homologue, mDelta1, expressed on feeder cells controls cellular differentiation. *Cell Structure and Function* **25,** 21.

Moloney, D. J., Shair, L. H., Lu, F. M., Xia, J. *et al.* (2000). Mammalian Notch1 is modified with two unusual forms of O-linked glycosylation found on epidermal growth factor-like modules. *J Biol Chem* **275,** 9604.

Moore, K. W., de Waal-Malefyt, R., Coffman, R. L., and O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. *Ann Rev Immunol* **19,** 683.

Morelli, A. E., O'Connell, P. J., Khanna, A., Logar, A. J. *et al.* (2000). Preferential induction of Th1 responses by functionally mature hepatic (CD8alpha- and CD8alpha+) dendritic cells: association with conversion from liver transplant tolerance to acute rejection. *Transplantation* **69,** 2647.

Morimura, T., Goitsuka, R., Zhang, Y., Saito, I. *et al.* (2000). Cell cycle arrest and apoptosis induced by Notch1 in B cells. *J Biol Chem* **275,** 36523.

Morimura, T., Miyatani, S., Kitamura, D., and Goitsuka, R. (2001). Notch signaling suppresses IgH gene expression in chicken B cells: implication in spatially restricted expression of Serrate2/Notch1 in the bursa of Fabricius. *J Immunol* **166,** 3277.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. *et al.* (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* **136,** 2348.

Murphy, D. B., Rath, S., Pizzo, E., Rudensky, A. Y. *et al.* (1992). Monoclonal antibody detection of a major self peptide. MHC class II complex. *J Immunol* **148,** 3483.

Murphy, E., Shibuya, K., Hosken, N., Openshaw, P. *et al.* (1996). Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. *J Exp Med* **183,** 901.

Murphy, K. M., Ouyang, W., Farrar, J. D., Yang, J. *et al.* (2000). Signaling and transcription in T helper development. *Ann Rev Immunol* **18,** 451.

Murray, V. (1989). Improved double-stranded DNA sequencing using the linear polymerase chain reaction. *Nucleic Acids Res* **17,** 8889.

Muzio, M., Bosisio, D., Polentarutti, N., D'amico, G. *et al.* (2000). Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol* **164,** 5998.

Myones, B. L., Dalzell, J. G., Hogg, N., and Ross, G. D. (1988). Neutrophil and monocyte cell surface p150,95 has iC3b-receptor (CR4) activity resembling CR3. *J Clin Invest* **82,** 640.

Nelms, K., Keegan, A. D., Zamorano, J., Ryan, J. J. *et al.* (1999). The IL-4 receptor: signaling mechanisms and biologic functions. *Ann Rev Immunol* **17,** 701.

Ng, W. F., Duggan, P. J., Ponchel, F., Matarese, G. *et al.* (2001). Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood* **98,** 2736.

Nofziger, D., Miyamoto, A., Lyons, K. M., and Weinmaster, G. (1999). Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. *Development* **126,** 1689.

Nomaguchi, K., Suzu, S., Yamada, M., Hayasawa, H. *et al.* (2001). Expression of Jagged1 gene in macrophages and its regulation by hematopoietic growth factors. *Experimental Hematology* **29,** 850.

Norcross, M. A., Bentley, D. M., Margulies, D. H., and Germain, R. N. (1984). Membrane Ia expression and antigen-presenting accessory cell function of L cells transfected with class II major histocompatibility complex genes. *J Exp Med* **160,** 1316.

Nussenzweig, M. C., and Steinman, R. M. (1980). Contribution of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction. *J Exp Med* **151,** 1196.

Obst, R., Munz, C., Stevanovi‡, S., and Rammensee, H. G. (1998). Allo- and self-restricted cytotoxic T lymphocytes against a peptide library: evidence for a functionally diverse allorestricted T cell repertoire. *Eur J Immunol* **28,** 2432.

Oda, T., Elkahloun, A. G., Meltzer, P. S., and Chandrasekharappa, S. C. (1997). Identification and cloning of the human homolog (JAG1) of the rat Jagged1 gene from the Alagille syndrome critical region at 20p12. *Genomics* **43,** 376.

O'Flaherty, E., Wong, W. K., Pettit, S. J., Seymour, K. *et al.* (2000). Regulation of T-cell apoptosis: a mixed lymphocyte reaction model. *Immunology* **100,** 289.

O'Garra, A. (1998). Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* **8,** 275.

O'Garra, A., Chang, R., Go, N., Hastings, R. *et al.* (1992). Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. *Eur J Immunol* **22,** 711.

Ohishi, K., Varnum-Finney, B., Flowers, D., Anasetti, C. *et al.* (2000). Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand, Delta-1. *Blood* **95,** 2847.

Ohishi, K., Varnum-Finney, B., Serda, R. E., Anasetti, C. *et al.* (2001). The Notch ligand, Delta-1, inhibits the differentiation of monocytes into macrophages but permits their differentiation into dendritic cells. *Blood* **98,** 1402.

Ohteki, T., Fukao, T., Suzue, K., Maki, C. *et al.* (1999). Interleukin 12-dependent interferon gamma production by CD8alpha+ lymphoid dendritic cells. *J Exp Med* **189,** 1981.

Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S. *et al.* (1999). Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. *EMBO J* **18,** 2196.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. *et al.* (1997). 'Green mice' as a source of ubiquitous green cells. *Febs Letters* **407,** 313.

Okazaki, T., Ozaki, S., and Nakao, K. (1994). CD4+ T cells require adhesion via LFA-1/ICAM-1 to induce target apoptosis in TNF-independent pathway. *Cell Immunol* **156,** 135.

Olweus, J., BitMansour, A., Warnke, R., Thompson, P. A. *et al.* (1997). Dendritic cell ontogeny: a human dendritic cell lineage of myeloid origin. *Proc Natl Acad Sci USA* **94,** 12551.

Ordentlich, P., Lin, A., Shen, C. P., Blaumueller, C. *et al.* (1998). Notch inhibition of E47 supports the existence of a novel signaling pathway. *Mol Cell Biol* **18,** 2230.

O'Rourke, R. W., Kang, S. M., Lower, J. A., Feng, S. *et al.* (2000). A dendritic cell line genetically modified to express CTLA4-IG as a means to prolong islet allograft survival. *Transplantation* **69,** 1440.

Oswald, F., Liptay, S., Adler, G., and Schmid, R. M. (1998). NF-kappaB2 is a putative target gene of activated Notch-1 via RBP-Jkappa. *Mol Cell Biol* **18,** 2077.

Ouyang, W., Jacobson, N. G., Bhattacharya, D., Gorham, J. D. *et al.* (1999). The Ets transcription factor ERM is Th1-specific and induced by IL-12 through a Stat4-dependent pathway. *Proc Natl Acad Sci USA* **96,** 3888.

Ouyang, W., Ranganath, S. H., Weindel, K., Bhattacharya, D. *et al.* (1998). Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. *Immunity* **9,** 745.

Pala, P., Hussell, T., and Openshaw, P. J. (2000). Flow cytometric measurement of intracellular cytokines. *J Immunol Methods* **243,** 107.

Palmieri, M., Sasso, M. P., Monese, R., Merola, M. *et al.* (1999). Interaction of the nuclear protein CBF1 with the kappaB site of the IL-6 gene promoter. *Nucleic Acids Res* **27,** 2785.

Palmiter, R. D., Chen, H. Y., and Brinster, R. L. (1982). Differential regulation of metallothionein-thymidine kinase fusion genes in transgenic mice and their offspring. *Cell* **29,** 701.

Pampeno, C. L., and Meruelo, D. (1996). A novel cDNA transcript expressed in fractionated X-irradiation-induced murine thymomas. *Cell Growth and Differentiation* **7,** 1113.

Panin, V. M., Papayannopoulos, V., Wilson, R., and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387,** 908.

Parada, N. A., Center, D. M., Kornfeld, H., Rodriguez, W. L. *et al.* (1998). Synergistic activation of CD4+ T cells by IL-16 and IL-2. *J Immunol* **160,** 2115.

Pelchen-Matthews, A., Parsons, I. J., and Marsh, M. (1993). Phorbol ester-induced downregulation of CD4 is a multistep process involving dissociation from p56lck, increased association with clathrin-coated pits, and altered endosomal sorting. *J Exp Med* **178,** 1209.

Piemonti, L., Monti, P., Allavena, P., Sironi, M. *et al.* (1999). Glucocorticoids affect human dendritic cell differentiation and maturation. *J Immunol* **162,** 6473.

Pieters, J. (2000). MHC class II-restricted antigen processing and presentation. *Adv Immunol* **75,** 159.

Pivniouk, V. I., and Geha, R. S. (2000). The role of SLP-76 and LAT in lymphocyte development. *Curr Opin Immunol* **12,** 173.

Poltorak, A., He, X., Smirnova, I., Liu, M. Y. *et al.* (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282,** 2085.

Prasher, D. C. (1995). Using GFP to see the light. *Trends Genet* **11,** 320.

Prickett, T. C., McKenzie, J. L., and Hart, D. N. (1988). Characterization of interstitial dendritic cells in human liver. *Transplantation* **46,** 754.

Pui, J. C., Allman, D., Xu, L., DeRocco, S. *et al.* (1999). Notch1expression in early lymphopoiesis influences B versus T lineage determination. *Immunity* **11,** 299.

Pulendran, B., Banchereau, J., Maraskovsky, E., and Maliszewski, C. (2001). Modulating the immune response with dendritic cells and their growth factors. *Trends Immunol* **22,** 41.

Pulendran, B., Smith, J. L., Caspary, G., Brasel, K. *et al.* (1999). Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc Natl Acad Sci USA* **96,** 1036.

Radtke, F., Ferrero, I., Wilson, A., Lees, R. *et al.* (2000). Notch1 deficiency dissociates the intrathymic development of dendritic cells and T cells. *J Exp Med* **191,** 1085.

Radtke, F., Wilson, A., Stark, G., Bauer, M. *et al.* (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10,** 547.

Raff, M. C. (1971). Surface antigenic markers for distinguishing T and B lymphocytes in mice. *Transplantation Reviews* **6,** 52.

Rao, A., Luo, C., and Hogan, P. G. (1997). Transcription factors of the NFAT family: regulation and function. *Ann Rev Immunol* **15,** 707.

Read, S., Malmstrom, V., and Powrie, F. (2000). Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* **192,** 295.

Rebay, I., Fehon, R. G., and Artavanis-Tsakonas, S. (1993). Specific truncations of *Drosophila* Notch define dominant activated and dominant negative forms of the receptor. *Cell* **74,** 319.

Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L. *et al.* (1991). Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* **67,** 687.

Rees, S., Coote, J., Stables, J., Goodson, S. *et al.* (1996). Bicistronic vector for the creation of stable mammalian cell lines that predisposes all antibiotic-resistant cells to express recombinant protein. *Biotechniques* **20,** 102.

Reid, C. D., Stackpoole, A., Meager, A., and Tikerpae, J. (1992). Interactions of tumor necrosis factor with granulocyte-macrophage colony-stimulating factor and other cytokines in the regulation of dendritic cell growth in vitro from early bipotent CD34+ progenitors in human bone marrow. *J Immunol* **149,** 2681.

Reiss, C. S., Evans, G. A., Margulies, D. H., Seidman, J. G. *et al.* (1983). Allospecific and virus-specific cytolytic T lymphocytes are restricted to the N or C1 domain of H-2 antigens expressed on L cells after DNA-mediated gene transfer. *Proc Natl Acad Sci USA* **80,** 2709.

Rengarajan, J., Szabo, S.J., and Glimcher, L.H (2000). Transcriptional regulation of Th1/Th2 polarization. *Immunol Today* **21**, 479.

Res, P., Martínez-Cáceres, E., Cristina Jaleco, A., Staal, F. *et al.* (1996). CD34+CD38dim cells in the human thymus can differentiate into T, natural killer, and dendritic cells but are distinct from pluripotent stem cells. *Blood* **87,** 5196.

Rescigno, M., Granucci, F., Citterio, S., Foti, M. *et al.* (1999). Coordinated events during bacteria-induced DC maturation. *Immunol Today* **20,** 200.

Richter, A., Lohning, M., and Radbruch, A. (1999). Instruction for cytokine expression in T helper lymphocytes in relation to proliferation and cell cycle progression. *J Exp Med* **190,** 1439.

Ridge, J. P., Di Rosa, F., and Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell [see comments]. *Nature* **393,** 474.

Rincon, M., Anguita, J., Nakamura, T., Fikrig, E. *et al.* (1997a). Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. *J Exp Med* **185,** 461.

Rincon, M., Derijard, B., Chow, C. W., Davis, R. J. *et al.* (1997b). Reprogramming the signalling requirement for AP-1 (activator protein-1) activation during differentiation of precursor CD4+ T-cells into effector Th1 and Th2 cells. *Genes Funct* **1,** 51.

Rissoan, M. C., Soumelis, V., Kadowaki, N., Grouard, G. *et al.* (1999). Reciprocal control of T helper cell and dendritic cell differentiation [see comments]. *Science* **283,** 1183.

Roake, J. A., Rao, A. S., Morris, P. J., Larsen, C. P. *et al.* (1995a). Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J Exp Med* **181,** 2237.

Roake, J. A., Rao, A. S., Morris, P. J., Larsen, C. P. *et al.* (1995b). Systemic lipopolysaccharide recruits dendritic cell progenitors to nonlymphoid tissues. *Transplantation* **59,** 1319.

Robbins, D. J., Zhen, E., Cheng, M., Xu, S. *et al.* (1994). MAP kinases ERK1 and ERK2: pleiotropic enzymes in a ubiquitous signaling network. *Advances in Cancer Research* **63,** 93.

Robert, C., Fuhlbrigge, R. C., Kieffer, J. D., Ayehunie, S. *et al.* (1999). Interaction of dendritic cells with skin endothelium: A new perspective on immunosurveillance [see comments]. *J Exp Med* **189,** 627.

Robey, E., Chang, D., Itano, A., Cado, D. *et al.* (1996). An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. *Cell* **87,** 483.

Robinson, D., Shibuya, K., Mui, A., Zonin, F. *et al.* (1997). IGIF does not drive Th1 development but synergizes with IL-12 for interferon-gamma production and activates IRAK and NFkappaB. *Immunity* **7,** 571.

Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234,** 364.

Romani, L., Mencacci, A., Cenci, E., Spaccapelo, R. *et al.* (1997). Neutrophil production of IL-12 and IL-10 in candidiasis and efficacy of IL-12 therapy in neutropenic mice. *J Immunol* **158,** 5349.

Romani, N., Gruner, S., Brang, D., Kampgen, E. *et al.* (1994). Proliferating dendritic cell progenitors in human blood. *J Exp Med* **180,** 83.

Romani, N., Reider, D., Heuer, M., Ebner, S. *et al.* (1996). Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. *J Immunol Methods* **196,** 137.

Romano, M. F., Lamberti, A., Petrella, A., Bisogni, R. *et al.* (1996). IL-10 inhibits nuclear factor-kappa B/Rel nuclear activity in CD3-stimulated human peripheral T lymphocytes. *J Immunol* **156,** 2119.

Ronchese, F., Brown, M. A., and Germain, R. N. (1987). Structure-function analysis of the Abm12 beta mutation using site-directed mutagenesis and DNA-mediated gene transfer. *J Immunol* **139,** 629.

Rudensky, A., and Janeway, C. A. (1993). Studies on naturally processed peptides associated with MHC class II molecules. *Chemical Immunology* **57,** 134.

Russell, J. H. (1995). Activation-induced death of mature T cells in the regulation of immune responses. *Curr Opin Immunol* **7,** 382.

Sad, S., Marcotte, R., and Mosmann, T. R. (1995). Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. *Immunity* **2,** 271.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. *et al.* (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* **155,** 1151.

Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products [see comments]. *J Exp Med* **182,** 389.

Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* **179,** 1109.

Sallusto, F., and Lanzavecchia, A. (2000). Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunol Rev* **177,** 134.

Sallusto, F., Schaerli, P., Loetscher, P., Schaniel, C. *et al.* (1998). Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* **28,** 2760.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning. A laboratory manual. 2nd edition" , Cold Spring Harbor Laboratory Press, New York.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74,** 5463.

Sanlioglu, S., Williams, C. M., Samavati, L., Butler, N. S. *et al.* (2001). Lipopolysaccharide induces Rac1-dependent reactive oxygen species formation and coordinates tumor necrosis factor-alpha secretion through IKK regulation of NF-kappa B. *J Biol Chem* **276,** 30188.

Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. *et al.* (1992). Two mammalian helix-loop-helix factors structurally related to *Drosophila* hairy and Enhancer of split. *Genes Dev* **6,** 2620.

Sato, K., Nagayama, H., Tadokoro, K., Juji, T. *et al.* (1999). Extracellular signal-regulated kinase, stress-activated protein kinase/c-Jun N-terminal kinase, and p38mapk are involved in IL-10-mediated selective repression of TNF-alpha-induced activation and maturation of human peripheral blood monocyte-derived dendritic cells. *J Immunol* **162,** 3865.

Scheicher, C., Mehlig, M., Zecher, R., and Reske, K. (1992). Dendritic cells from mouse bone marrow: in vitro differentiation using low doses of recombinant granulocyte-macrophage colony-stimulating factor. *J Immunol Methods* **154,** 253.

Schlissel, M., Voronova, A., and Baltimore, D. (1991). Helix-loop-helix transcription factor E47 activates germ-line immunoglobulin heavy-chain gene transcription and rearrangement in a pre-T-cell line. *Genes Dev* **5,** 1367.

Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R. *et al.* (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* **393,** 480.

Schottelius, A. J., Mayo, M. W., Sartor, R. B., and Baldwin, A. S. (1999). Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. *J Biol Chem* **274,** 31868.

Schroeder, T., and Just, U. (2000a). mNotch1 signaling reduces proliferation of myeloid progenitor cells by altering cell-cycle kinetics. *Exp Hematol* **28,** 1206.

Schroeder, T., and Just, U. (2000b). Notch signalling via RBP-J promotes myeloid differentiation. *EMBO J* **19,** 2558.

Schuldt, A. J., and Brand, A. H. (1999). Mastermind acts downstream of notch to specify neuronal cell fates in the *Drosophila* central nervous system. *Dev Biol* **205,** 287.

Shawber, C., Nofziger, D., Hsieh, J. J., Lindsell, C. *et al.* (1996). Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. *Development* **122,** 3765.

Shibaki, A., Meunier, L., Ra, C., Shimada, S. *et al.* (1995). Differential responsiveness of Langerhans cell subsets of varying phenotypic states in normal human epidermis. *J Invest Dermatol* **104,** 42.

Shimizu, K., Chiba, S., Hosoya, N., Kumano, K. *et al.* (2000). Binding of Delta1, Jagged1, and Jagged2 to Notch2 rapidly induces cleavage, nuclear translocation, and hyperphosphorylation of Notch2. *Mol Cell Biol* **20,** 6913.

Shimizu, K., Chiba, S., Kumano, K., Hosoya, N. *et al.* (1999). Mouse jagged1 physically interacts with notch2 and other notch receptors. Assessment by quantitative methods. *J Biol Chem* **274,** 32961.

Shimonkevitz, R., Bar-Or, D., Harris, L., Northrop, J. *et al.* (2000). Granulocytes, including neutrophils, synthesize IL-10 after traumatic pancreatitis: case report. *Journal of Trauma* **48,** 165.

Small, D., Kovalenko, D., Kacer, D., Liaw, L. *et al.* (2001). Soluble Jagged 1 represses the function of its transmembrane form to induce the formation of the Src-dependent chord-like phenotype. *J Biol Chem* **276,** 32022.

Smith, P. A., Brunmark, A., Jackson, M. R., and Potter, T. A. (1997). Peptide-independent recognition by alloreactive cytotoxic T lymphocytes (CTL). *J Exp Med* **185,** 1023.

Song, J. S., Haleem-Smith, H., Arudchandran, R., Gomez, J. *et al.* (1999a). Tyrosine phosphorylation of Vav stimulates IL-6 production in mast cells by a Rac/c-Jun N-terminal kinase-dependent pathway. *J Immunol* **163,** 802.

Song, W., Nadeau, P., Yuan, M., Yang, X. *et al.* (1999b). Proteolytic release and nuclear translocation of Notch-1 are induced by presenilin-1 and impaired by pathogenic presenilin-1 mutations. *Proc Natl Acad Sci USA* **96,** 6959.

Sornasse, T., Flamand, V., De Becker, G., Bazin, H. *et al.* (1992). Antigen-pulsed dendritic cells can efficiently induce an antibody response in vivo. *J Exp Med* **175,** 15.

Sozzani, S., Allavena, P., Vecchi, A., and Mantovani, A. (1999). The role of chemokines in the regulation of dendritic cell trafficking. *Journal of Leukocyte Biology* **66,** 1.

Sozzani, S., Sallusto, F., Luini, W., Zhou, D. *et al.* (1995). Migration of dendritic cells in response to formyl peptides, C5a, and a distinct set of chemokines. *J Immunol* **155,** 3292.

Spencer, S. C., and Fabre, J. W. (1990). Characterization of the tissue macrophage and the interstitial dendritic cell as distinct leukocytes normally resident in the connective tissue of rat heart. *J Exp Med* **171,** 1841.

Sperling, A. I., Auger, J. A., Ehst, B. D., Rulifson, I. C. *et al.* (1996). CD28/B7 interactions deliver a unique signal to naive T cells that regulates cell survival but not early proliferation. *J Immunol* **157,** 3909.

Sredni, B., and Schwartz, R. H. (1981). Antigen-specific, proliferating T lymphocyte clones. Methodology, specificity, MHC restriction and alloreactivity. *Immunol Rev* **54,** 187.

Starling, G. C., McLellan, A. D., Egner, W., Sorg, R. V. *et al.* (1995). Intercellular adhesion molecule-3 is the predominant co-stimulatory ligand for leukocyte function antigen-1 on human blood dendritic cells. *Eur J Immunol* **25,** 2528.

Steinbrink, K., Jonuleit, H., Muller, G., Schuler, G. *et al.* (1999). Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. *Blood* **93,** 1634.

Steinbrink, K., Wölfl, M., Jonuleit, H., Knop, J. *et al.* (1997). Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* **159,** 4772.

Steinman, R. M. (1991). The dendritic cell system and its role in immunogenicity. *Ann Rev Immunol* **9,** 271.

Steinman, R. M., Lustig, D. S., and Cohn, Z. A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. 3. Functional properties in vivo. *J Exp Med* **139,** 1431.

Stinchcombe, J. C., and Griffiths, G. M. (2001). Normal and abnormal secretion by haemopoietic cells. *Immunology* **103,** 10.

Stockinger, B., Zal, T., Zal, A., and Gray, D. (1996). B cells solicit their own help from T cells. *J Exp Med* **183,** 891.

Straus, D. B., and Weiss, A. (1992). Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell* **70,** 585.

Strobl, H., and Knapp, W. (1999). TGF-beta1 regulation of dendritic cells. *Microbes Infect* **1,** 1283.

Struhl, G., and Greenwald, I. (1999). Presenilin is required for activity and nuclear access of Notch in *Drosophila* [see comments]. *Nature* **398,** 522.

Stumbles, P. A., Thomas, J. A., Pimm, C. L., Lee, P. T. *et al.* (1998). Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity. *J Exp Med* **188,** 2019.

Su, B., Jacinto, E., Hibi, M., Kallunki, T. *et al.* (1994). JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* **77,** 727.

Sun, J., McCaughan, G. W., Gallagher, N. D., Sheil, A. G. *et al.* (1995). Deletion of spontaneous rat liver allograft acceptance by donor irradiation. *Transplantation* **60,** 233.

Suss, G., and Shortman, K. (1996). A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. *J Exp Med* **183,** 1789.

Szabo, S. J., Dighe, A. S., Gubler, U., and Murphy, K. M. (1997). Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med* **185,** 817.

Szabo, S. J., Jacobson, N. G., Dighe, A. S., Gubler, U. *et al.* (1995). Developmental commitment to the Th2 lineage by extinction of IL-12 signaling. *Immunity* **2,** 665.

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X. *et al.* (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100,** 655.

Taga, K., Mostowski, H., and Tosato, G. (1993). Human interleukin-10 can directly inhibit T-cell growth. *Blood* **81,** 2964.

Takahashi, T., Tagami, T., Yamazaki, S., Uede, T. *et al.* (2000). Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* **192,** 303.

Takayama, T., Nishioka, Y., Lu, L., Lotze, M. T. *et al.* (1998). Retroviral delivery of viral interleukin-10 into myeloid dendritic cells markedly inhibits their allostimulatory activity and promotes the induction of T-cell hyporesponsiveness. *Transplantation* **66,** 1567.

Takenaka, H., Maruo, S., Yamamoto, N., Wysocka, M. *et al.* (1997). Regulation of T cell-dependent and -independent IL-12 production by the three Th2-type cytokines IL-10, IL-6, and IL-4. *Journal of Leukocyte Biology* **61,** 80.

Talmage, D. W., Dart, G., Radovich, J., and Lafferty, K. J. (1976). Activation of transplant immunity: effect of donor leukocytes on thyroid allograft rejection. *Science* **191,** 385.

Tamura, K., Taniguchi, Y., Minoguchi, S., Sakai, T. *et al.* (1995). Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H). *Curr Biol* **5,** 1416.

Tang, A., Amagai, M., Granger, L. G., Stanley, J. R. *et al.* (1993). Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature* **361,** 82.

Tang, H. L., and Cyster, J. G. (1999). Chemokine Up-regulation and activated T cell attraction by maturing dendritic cells. *Science* **284,** 819.

Tan-Pertel, H. T., Walker, L., Browning, D., Miyamoto, A. *et al.* (2000). Notch signaling enhances survival and alters differentiation of 32D myeloblasts. *J Immunol* **165,** 4428.

te Riele, H., Maandag, E. R., and Berns, A. (1992). Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proc Natl Acad Sci USA* **89,** 5128.

Thanhauser, A., Reiling, N., Bohle, A., Toellner, K. M. *et al.* (1993). Pentoxifylline: a potent inhibitor of IL-2 and IFN-gamma biosynthesis and BCG-induced cytotoxicity. *Immunology* **80,** 151.

Thery, C., and Amigorena, S. (2001). The cell biology of antigen presentation in dendritic cells. *Curr Opin Immunol* **13,** 45.

Thompson, C. B., and Allison, J. P. (1997). The emerging role of CTLA-4 as an immune attenuator. *Immunity* **7,** 445.

Thomson, A. W., and Lu, L. (1999). Are dendritic cells the key to liver transplant tolerance? *Immunol Today* **20,** 27.

Thomson, A. W., Lu, L., Subbotin, V. M., Li, Y. *et al.* (1995). In vitro propagation and homing of liver-derived dendritic cell progenitors to lymphoid tissues of allogeneic recipients. Implications for the establishment and maintenance of donor cell chimerism following liver transplantation. *Transplantation* **59,** 544.

Thomson, A. W., and Takayama, T. (1999). Dendritic cells and the outcome of organ transplantation: a contemporary view. *Transplant Proc* **31,** 2738.

Thornton, A. M., and Shevach, E. M. (1998). CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* **188,** 287.

Tomita, K., Hattori, M., Nakamura, E., Nakanishi, S. *et al.* (1999). The bHLH gene Hes1 is essential for expansion of early T cell precursors. *Genes Dev* **13,** 1203.

Toungouz, M., Denys, C., de Groote, D., Andrien, M. *et al.* (1996). Optimal control of interferon-gamma and tumor necrosis factor-alpha by interleukin-10 produced in response to one HLA-DR mismatch during the primary mixed lymphocyte reaction. *Transplantation* **61,** 497.

Traver, D., Akashi, K., Manz, M., Merad, M. *et al.* (2000). Development of CD8alpha-positive dendritic cells from a common myeloid progenitor. *Science* **290,** 2152.

Trinchieri, G. (1995). Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Ann Rev Immunol* **13,** 251.

Troy, A., Davidson, P., Atkinson, C., and Hart, D. (1998). Phenotypic characterisation of the dendritic cell infiltrate in prostate cancer [see comments]. *Journal Of Urology* **160,** 214.

Tsai, S., Fero, J., and Bartelmez, S. (2000). Mouse Jagged2 is differentially expressed in hematopoietic progenitors and endothelial cells and promotes the survival and proliferation of hematopoietic progenitors by direct cell-to-cell contact. *Blood* **96,** 950.

Umlauf, S. W., Beverly, B., Lantz, O., and Schwartz, R. H. (1995). Regulation of interleukin 2 gene expression by CD28 costimulation in mouse T-cell clones: both nuclear and cytoplasmic RNAs are regulated with complex kinetics. *Mol Cell Biol* **15,** 3197.

Usherwood, E. J., Crowther, G., and Woodland, D. L. (1999). Apoptotic cells are generated at every division of in vitro cultured T cell lines. *Cell Immunol* **196,** 131.

Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q. *et al.* (1996). Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. *Development* **122,** 2251.

Valitutti, S., Muller, S., Salio, M., and Lanzavecchia, A. (1997). Degradation of T cell receptor (TCR)-CD3-zeta complexes after antigenic stimulation. *J Exp Med* **185,** 1859.

Van Parijs, L., and Abbas, A. K. (1996). Role of Fas-mediated cell death in the regulation of immune responses. *Curr Opin Immunol* **8,** 355.

Varnum-Finney, B., Purton, L. E., Yu, M., Brashem-Stein, C. *et al.* (1998). The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. *Blood* **91,** 4084.

Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C. *et al.* (2000). Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nat Med* **6,** 1278.

Veerman, A. J., and van Ewijk, W. (1975). White pulp compartments in the spleen of rats and mice. A light and electron microscopic study of lymphoid and non-lymphoid celltypes in T- and B-areas. *Cell and Tissue Research* **156,** 417.

Veiga-Fernandes, H., Walter, U., Bourgeois, C., McLean, A. *et al.* (2000). Response of naive and memory CD8+ T cells to antigen stimulation in vivo. *Nat Immunol* **1,** 47.

Vendetti, S., Chai, J. G., Dyson, J., Simpson, E. *et al.* (2000). Anergic T cells inhibit the antigen-presenting function of dendritic cells. *J Immunol* **165,** 1175.

Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* **184,** 39.

Villalba, M., Hernandez, J., Deckert, M., Tanaka, Y. *et al.* (2000). Vav modulation of the Ras/MEK/ERK signaling pathway plays a role in NFAT activation and CD69 up-regulation. *Eur J Immunol* **30,** 1587.

Viola, A., Schroeder, S., Sakakibara, Y., and Lanzavecchia, A. (1999). T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* **283,** 680.

Vremec, D., and Shortman, K. (1997). Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes with incubation, and differences among thymus, spleen, and lymph nodes. *J Immunol* **159,** 565.

Vremec, D., Zorbas, M., Scollay, R., Saunders, D. J. *et al.* (1992). The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J Exp Med* **176,** 47.

Wakkach, A., Cottrez, F., and Groux, H. (2001). Differentiation of regulatory t cells 1 is induced by cd2 costimulation. *J Immunol* **167,** 3107.

Waldmann, T. A., and Tagaya, Y. (1999). The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Ann Rev Immunol* **17,** 19.

Walker, L., Lynch, M., Silverman, S., Fraser, J. *et al.* (1999). The Notch/Jagged pathway inhibits proliferation of human hematopoietic progenitors in vitro. *Stem Cells* **17,** 162.

Walunas, T. L., Lenschow, D. J., Bakker, C. Y., Linsley, P. S. *et al.* (1994). CTLA-4 can function as a negative regulator of T cell activation. *Immunity* **1,** 405.

Wang, B., Maile, R., Greenwood, R., Collins, E. J. *et al.* (2000). Naive CD8+ T cells do not require costimulation for proliferation and differentiation into cytotoxic effector cells. *J Immunol* **164,** 1216.

Wang, J., Shelly, L., Miele, L., Boykins, R. *et al.* (2001). Human notch-1 inhibits nf-kappab activity in the nucleus through a direct interaction involving a novel domain. *J Immunol* **167,** 289.

Wang, P., Wu, P., Siegel, M. I., Egan, R. W. *et al.* (1995). Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. *J Biol Chem* **270,** 9558.

Ward, D. M., Griffiths, G. M., Stinchcombe, J. C., and Kaplan, J. (2000). Analysis of the lysosomal storage disease Chediak-Higashi syndrome. *Traffic* **1,** 816.

Washburn, T., Schweighoffer, E., Gridley, T., Chang, D. *et al.* (1997). Notch activity influences the alphabeta versus gammadelta T cell lineage decision. *Cell* **88,** 833.

Weber, D. A., Terrell, N. K., Zhang, Y., Strindberg, G. *et al.* (1995). Requirement for peptide in alloreactive CD4+ T cell recognition of class II MHC molecules. *J Immunol* **154,** 5153.

Weiss, A. (1993). T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. *Cell* **73,** 209.

Weiss, A., and Imboden, J. B. (1987). Cell surface molecules and early events involved in human T lymphocyte activation. *Adv Immunol* **41,** 1.

Whelan, M., Harnett, M. M., Houston, K. M., Patel, V. *et al.* (2000). A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells. *J Immunol* **164,** 6453.

Wilbanks, G. A., and Streilein, J. W. (1992). Fluids from immune privileged sites endow macrophages with the capacity to induce antigen-specific immune deviation via a mechanism involving transforming growth factor-beta. *Eur J Immunol* **22,** 1031.

Wilson, A., Ferrero, I., MacDonald, H. R., and Radtke, F. (2000). Cutting edge: an essential role for Notch-1 in the development of both thymus-independent and -dependent T cells in the gut. *J Immunol* **165,** 5397.

Winzler, C., Rovere, P., Rescigno, M., Granucci, F. *et al.* (1997). Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J Exp Med* **185,** 317.

Wolfer, A., Bakker, T., Wilson, A., Nicolas, M. *et al.* (2001). Inactivation of Notch 1 in immature thymocytes does not perturb CD4 or CD8T cell development. *Nat Immunol* **2,** 235.

Wong, B. R., Josien, R., Lee, S. Y., Sauter, B. *et al.* (1997). TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J Exp Med* **186,** 2075.

Wong, M. K., Prudovsky, I., Vary, C., Booth, C. *et al.* (2000). A non-transmembrane form of Jagged-1 regulates the formation of matrix-dependent chord-like structures. *Biochem Biophys Res Commun* **268,** 853.

Woronicz, J. D., Calnan, B., Ngo, V., and Winoto, A. (1994). Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. *Nature* **367,** 277.

Wu, L., Aster, J. C., Blacklow, S. C., Lake, R. *et al.* (2000). MAML1, a human homologue of *Drosophila* mastermind, is a transcriptional co-activator for NOTCH receptors. *Nature Genetics* **26,** 484.

Wu, L., Li, C. L., and Shortman, K. (1996). Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. *J Exp Med* **184,** 903.

Wu, L., Vremec, D., Ardavin, C., Winkel, K. *et al.* (1995). Mouse thymus dendritic cells: kinetics of development and changes in surface markers during maturation. *Eur J Immunol* **25,** 418.

Wulfing, C., Rabinowitz, J. D., Beeson, C., Sjaastad, M. D. *et al.* (1997). Kinetics and extent of T cell activation as measured with the calcium signal. *J Exp Med* **185,** 1815.

Wurst, W., and Joyner, A. L. (1993). "Gene Targeting: A Practical Approach" , IRL Press, Oxford.

Yamada, A., Kishimoto, K., Dong, V. M., Sho, M. *et al.* (2001). CD28-independent costimulation of T cells in alloimmune responses. *J Immunol* **167,** 140.

Yamaguchi, Y., Tsumura, H., Miwa, M., and Inaba, K. (1997). Contrasting effects of TGF-beta 1 and TNF-alpha on the development of dendritic cells from progenitors in mouse bone marrow. *Stem Cells* **15,** 144.

Yang, J. S., Xu, L. Y., Huang, Y. M., Van Der Meide, P. H. *et al.* (2000). Adherent dendritic cells expressing high levels of interleukin-10 and low levels of interleukin-12 induce antigen-specific tolerance to experimental autoimmune encephalomyelitis. *Immunology* **101,** 397.

Yasutomo, K., Doyle, C., Miele, L., and Germain, R. N. (2000). The duration of antigen receptor signalling determines CD4+ versus CD8+ T-cell lineage fate. *Nature* **404,** 506.

Yellin, M. J., Winikoff, S., Fortune, S. M., Baum, D. *et al.* (1995). Ligation of CD40 on fibroblasts induces CD54 (ICAM-1) and CD106 (VCAM-1) up-regulation and IL-6 production and proliferation. *Journal of Leukocyte Biology* **58,** 209.

Yoneya, T., Tahara, T., Nagao, K., Yamada, Y. *et al.* (2001). Molecular cloning of delta-4, a new mouse and human Notch ligand. *J Biochem* **129,** 27.

Young, J. W., and Steinman, R. M. (1990). Dendritic cells stimulate primary human cytolytic lymphocyte responses in the absence of CD4+ helper T cells. *J Exp Med* **171,** 1315.

Zecchini, V., Brennan, K., and Martinez-Arias, A. (1999). An activity of Notch regulates JNK signalling and affects dorsal closure in *Drosophila*. *Curr Biol* **9,** 460.

Zenner, G., Vorherr, T., Mustelin, T., and Burn, P. (1996). Differential and multiple binding of signal transducing molecules to the ITAMs of the TCR-zeta chain. *Journal of Cellular Biochemistry* **63,** 94.

Zheng, W., and Flavell, R. A. (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* **89,** 587.

Zhou, L. J., and Tedder, T. F. (1996). CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells. *Proc Natl Acad Sci USA* **93,** 2588.

Zuckerman, L. A., Pullen, L., and Miller, J. (1998). Functional consequences of costimulation by ICAM-1 on IL-2 gene expression and T cell activation. *J Immunol* **160,** 3259.